EFFECT OF THE DIET ON THE LEVELS OF CAROTENOIDS, RETINOIDS AND ALPHA-TOCOPHEROL IN BIOLOGICAL FLUIDS AND TISSUES FROM ANIMALS OF DIFFERENT SPECIES.

Rocío Álvarez Alonso

“Effect of the diet on the levels of carotenoids, retinol and alpha-tocopherol in biological fluids and tissues from animals of different species”

Rocío Álvarez Alonso

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A mi abuelo

A Marisol

Caminante no hay camino, se hace camino al andar

(Antonio Machado)

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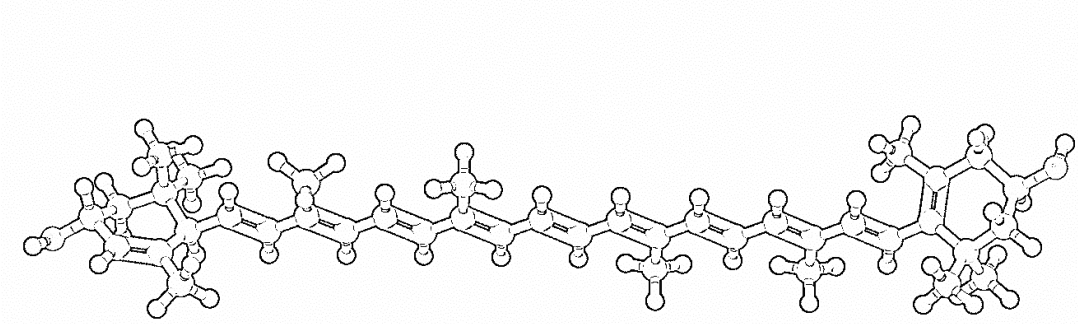
ABSTRACT



ABSTRACT

In this doctoral thesis, we have investigated the effect of different diets on the levels of carotenoids, retinoids and α-tocopherol in biological tissues of different livestock species (goats, sheep, pigs, horse and cattle). Carotenoids and fat-soluble vitamins, as retinol and tocopherol, may confer healthy properties to animal products and, in this way, improve consumer point of view about them. They are provided in the typical diets of the animals reared on extensive production systems, which are better accepted by the consumers. In addition, mammals can absorb and metabolize some of these compounds, being the diet one of the main factors that affect carotenoids, retinoids and tocopherol content in animal tissues. Although, species-specific differences also exist among livestock species.

Plasma and milk samples of goats fed on three different carotenoid-containing diets (pasture, concentrate and a diet enriched with orange pulp) were analyzed. The diets as well as their effect on carotenoids and retinol content in the selected tissues were studied. In this sense, it was observed that milk from goats fed on a diet rich in carotenoids (i.e., based on pasture or supplemented with orange pulp) showed higher ($P<0.001$) retinol levels than the milk from goats with a diet based on concentrate (1.18 ± 0.57, 1.02 ± 0.43 and 0.23 ± 0.18 µg/mL, respectively).

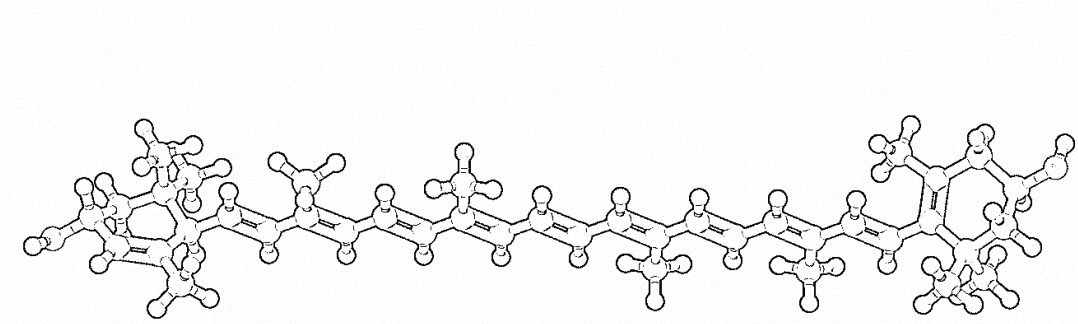
Plasma, adipose tissue and milk were the biological samples selected in the case of sheep. The effect of the diet on the carotenoids, retinol and α-tocopherol was also assessed in relation to the production system. Lutein (4.95 ± 0.71 µg/100 mL) and β-carotene (2.97 ± 0.68 µg/100 mL) content in plasma appeared as useful parameters to differentiate lambs fed exclusively with pasture from lambs fed on other diets, like concentrate and sheep milk (suckling lambs). The usefulness of the levels of the compounds studied in adipose tissue in combination with colour measurements was also evaluated in order to

differentiate stall-fed from pasture-fed lambs. A good discrimination (100% of the animals accurately classified into their group) of lambs according to their diet (pasture *vs.* concentrate) was obtained when a combination of AVI (integral of the reflectance translated spectrum), retinol and α-tocopherol levels in perirenal fat was used. Thus, these parameters are proposed as a useful tool for feeding traceability studies in sheep livestock.

Iberian pigs fed on two rearing systems: *Montanera* (extensive system where animals are fed on pasture and acorns) and *Cebo* (intensive system where animals are fed on concentrate) were compared. Both systems showed a different carotenoid profile (*P<0.01*). It was observed that plasma retinol (1.07 ± 0.69 µg/mL in pigs reared in extensive and 0.68 ± 0.38 µg/mL in pigs reared in intensive) and perirenal fat retinol content (4.14 ± 1.47 µg/g in pigs reared in extensive and 3.69 ± 0.77 µg/g in pigs reared in intensive) in these animals is not significantly affected by the different diets tested. However, retinoids content in liver, as well as L* and h_{ab} values in perirenal fat appeared as useful variables to differentiate these animals according to their feeding system (92.9% and 78.6% of the animals correctly classified, respectively).

Finally, the level of carotenoids, retinoids and α-tocopherol were assessed in horse and cattle tissues (plasma, milk, perirenal fat and liver). The animals were fed on different systems (outdoors system based on pasture and indoors based on concentrate). Cattle was selected as a reference model to which compare horse with. In this way, species-specific differences were observed in the accumulation of carotenoids, retinoids and α-tocopherol between equine and cattle livestock. In addition, horse and cattle seemed to have different metabolism for retinoids in liver, such that a higher (*P<0.001, P<0.01*) accumulation was found in the foal under our experimental conditions.

RESUMEN



RESUMEN

En la presente tesis doctoral hemos estudiado el efecto de diferentes dieras en los niveles de carotenoides, retinoides y α-tocoferol en tejidos biológicos que diferentes especies ganaderas (caprino, ovino, porcino, vacuno y equino). Tanto los carotenoides como las vitaminas liposolubles, entre ellas el retinol y el tocoferol, pueden conferir propiedades saludables a los productos animales y, así, mejorar el punto de vista de los consumidores respecto a ellos. Estos compuestos se encuentran presentes en las dietas de los animales críados en sistemas extensivos, los cuales están más valorados por los consumidores que los intensivos. Además, los mamíferos pueden absorber y metabolizar algunos carotenoides, siendo la dieta uno de los factores más importantes que afecta al contenido de carotenoides, retinoides y tocoferol en tejidos animales. Aunque no todas las especies de animales absorben y metabolizan estos compuestos de igual manera ya que existen diferencias específicas entre especies ganaderas al respecto.

Se analizó el efecto de tres dietas distintas respecto a su contenido en carotenoides (pasto, concentrado y una dieta enriquecida con pulpa de naranja) en los niveles de retinol y carotenoides en plasma y leche de ganado caprino. Se observaron niveles más altos ($P<0,001$) de reinol en la leche de cabras con una dieta rica en carotenoides (dieta basada en pastos o enriquecida con pulpa de naranja) que en la de aquellas con una dieta en estublación a base de concentrado (1,18 ± 0,57, 1,02 ± 0,43 y 0,23 ± 0,18 µg/mL, respectivamente).

En el caso del ganado ovino los tejidos y fluidos biológicos analizados fueron plasma, grasa y leche. Se estimó el efecto de la dieta sobre los niveles de carotenoides, retinol y α-tocoferol en relación al sistema de producción. Los niveles de luteína (4,95 ± 0,71 µg/100 mL) y de β-caroteno (2,97 ± 0,68 µg/100 mL) en plasma se mostraron como una herramienta útil para diferenciar a los corderos alimentados exclusivamente con pasto de

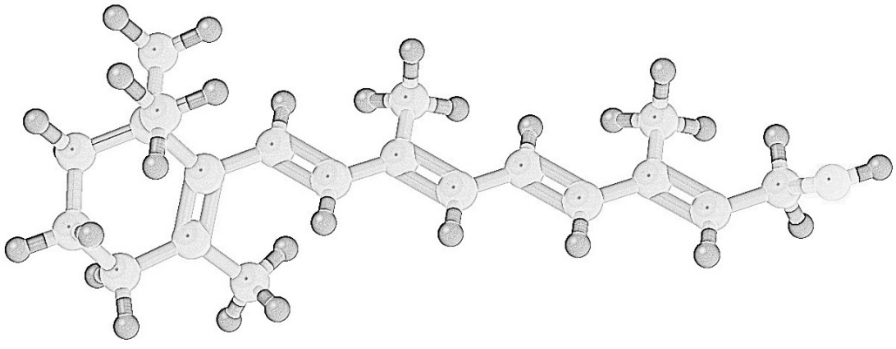
aquellos alimentados con otras dietas, como alimentos concentrados o leche (corderos lechales). También se estudió la posibilidad de combinar los niveles de carotenoides, retinoides y α-tocoferol en tejido adiposo con las medidas de los parámetros de color de dicho tejido para diferenciar los corderos estabulados con una dieta a base de pienso de los corderos alimentados exclusivamente con pasto. Utilizando una combinación de la medida de AVI (integral del espectro de reflectancia trasladado) junto con los niveles de retinol y α-tocoferol en grasa perirrenal, se discriminaron el 100% de los corderos según sus dieta (pastp *vs.* concentrado).

Se compararon dos grupos de cerdo ibérico con dos sistemas de producción disitntos: *Montanera* (sistema extensivo en el que los cerdos son alimentados con pasto y bellotas) y *Cebo* (sistema intensivo en el que los animales son alimentados con concentrados). Ambos sistemas de cría presentaron distintos niveles de carotenoides ($P<0,01$) en la alimentación de los animales. Sin embargo, los resultados obtenidos demuestran que los niveles de retinol en plasma (1,07 ± 0,69 µg/mL y 0,68 ± 0,38 µg/mL, montanera y cebo respectivamente) y grasa perirrenal (4,14 ± 1,47 µg/g y 3,69 ± 0,77 µg/g, montanera y cebo respectivamente) en cerdos ibéricos no se ven afectados por las dos dietas estudiadas. Sin embargo, tanto el contenido en retinoides en le hígado vomo los valores de L* y h_{ab} en grasa perirrenal resultaron útiles para diferenciar estso animales según sus sistema de cría (92,9% y 78,6% de los animales correctamente clasificados, respectivamente).

Por último, se estudiaron los niveles de carotenoides, retinoides y α-tocoferol en ganado bovino y equino (plasma, leche, grasa perirrenal e hígado). Estos animales fueron críados en dos sistemas diferentes: sistema extensivo con una dieta en base al pastoreo y sistema intensivo con una dieta en base a concentrado. El ganado bovino se utilizó como un modelo de referencia con el que comparar al ganado equino. De esta forma, se

evidenciaron diferencias específicas por especie en la acumulación de carotenoides, retinoides y α-tocoferol entre el ganado bovino y el equino. Además, el caballo y el vacuno parecen tener un metabolismo disinto para los retinoides en el hígado, ya que se observó un almacenamiento mayor ($P<0,001$, $P<0,01$) en potros que en terneros, bajo las mismas condicones.

INTEREST AND JUSTIFICATION



INTEREST AND JUSTIFICATION

Nowadays, there is an increasing interest of consumers about foods with additional health promoting functions (Olmedilla-Alonso et al., 2013). In this sense, consumers are focusing on the healthy properties of products from animals fed on pasture, which are considered as more suitable with a "green-image" of the animal production (Dian et al., 2007a; Nozière et al., 2006b; Prache et al., 2003b; Sheath et al., 2001). For these reasons, research on diet authentication in herbivore products is being conducted and efforts have recently been made to develop analytical tools to quantify specific compounds in animal´s tissues that can act as tracers of the conditions of production (Dian et al., 2007a; Engel et al., 2007). These compounds are transferred from the feed to the final product or transformed by the animal metabolism Thus, they could be used to some extent to authenticate the diet of the animals and, in this way, reassure consumers about the origin of the products and how they are produced (Dian et al., 2007b; Sheath et al., 2001). Carotenoids and fat-soluble vitamins, such as vitamins A (retinol) and E (α-tocopherol) are examples of these compounds. Although species-specific differences exist among livestock species, the diet is one of the main factors that affect the content of these compounds in animal tissues (Prache & Theriez, 1999; Schweigert, 1998). Considering all these facts, it is clear that a deeper knowledge of the factors governing the presence of these health-promoting compounds in biological fluids and tissues of different livestock species with different rearing systems is interesting.

European sheep and goat production represents, important economic, environmental and sociological issues, mainly in Mediterranean areas (De Rancourt et al., 2006). However, these productions have low profitability in these countries (Niżnikowski et al.,

2006), so the sector is in a critical economic situation and the production has decreased consistently over the last ten years in Europe (Figure 1).

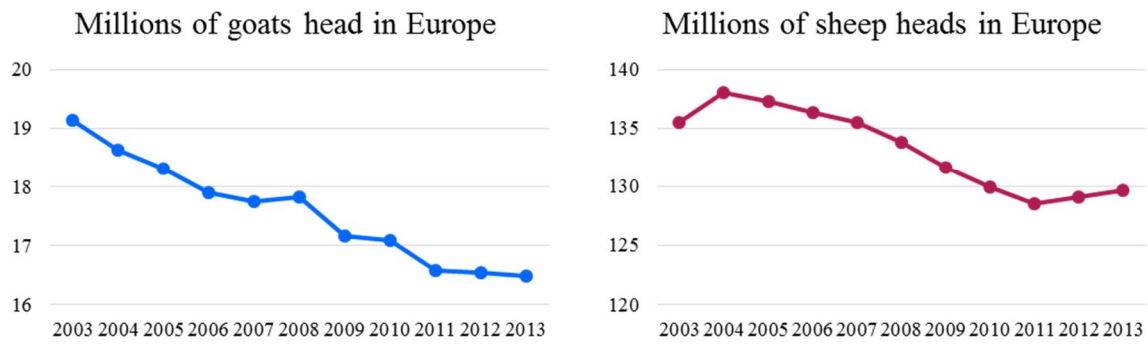


Figure 1. *Millions of goat and sheep heads in Europe between 2003 and 2013*
Graphics made from FAO (2014) data

One of the main reasons behind this problem is the decrease in the consumption due to limited availability of its products together with a relative high price, linked to high production costs (Alcalde et al., 2013; De Rancourt et al., 2006). Thus, to ensure a sustainable future for the small ruminant sector it is desirable to improve the consumption of its products. One strategy would be to add value to the milk, meat, etc. by increasing the content of health-promoting compounds (Nozière et al., 2006b; Prache & Theriez, 1999). Altogether, carotenoids, vitamins A and E, are involved in the nutritional and sensory properties of goat products, mainly in those from animals fed on pasture (Sauvant et al., 2012). Therefore, the study of biomarkers of the feeding system, such as carotenoids and fat-soluble vitamins in small ruminants products could be useful to find ways of conferring them added value related to health benefits and emphasizing their origin.

Figure 2. *Florida goat (left) and Segureña sheep (right) reared in an extensive system in Southwest of Spain*

On the other hand, the Iberian pig breed is one of the most important Mediterranean swine type, both in population size and economic importance (Juárez et al., 2009). According to the new Spanish legislation related to the quality standard for Iberian pig products (BOE, 2014) three different categories are considered based on feeding and rearing system. The highest quality products are related to the typical outdoor rearing system with a nutritional strategy based on acorns and grass (*Montanera*) (Gaspar et al., 2007). However, the controversy about the different feeding systems in this pig breed and the corresponding quality in derived products is still an unresolved problem. The traditional analysis of fatty acids has been proved to fail in this purpose. In this sense, other minor compounds related to the diet, like provitamin A carotenoids could be of interest to this aim.

Figure 3. *Iberian breed pig fed on Montanera rearing system in Southwestern Spain*

Finally, the situation of equine production in Europe, as well as the economic crisis in most of the South-European countries are deriving in an increment in the slaughters due to the inability of producers to assume the high production costs (Figure 4). Furthermore, mare's milk production is gaining importance in European countries because of its composition, since it can be a profitable alternative to cow's milk especially among people with cow's allergy. In addition, it is being used for cosmetic purposes (Markiewicz-Kęszycka et al., 2014).

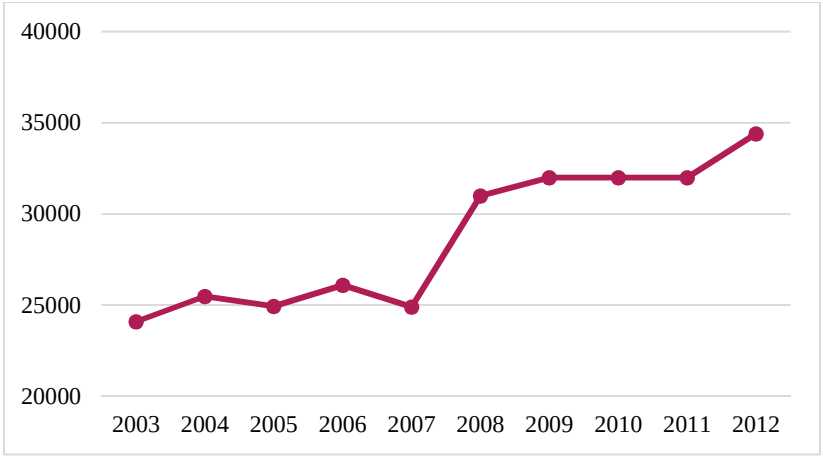


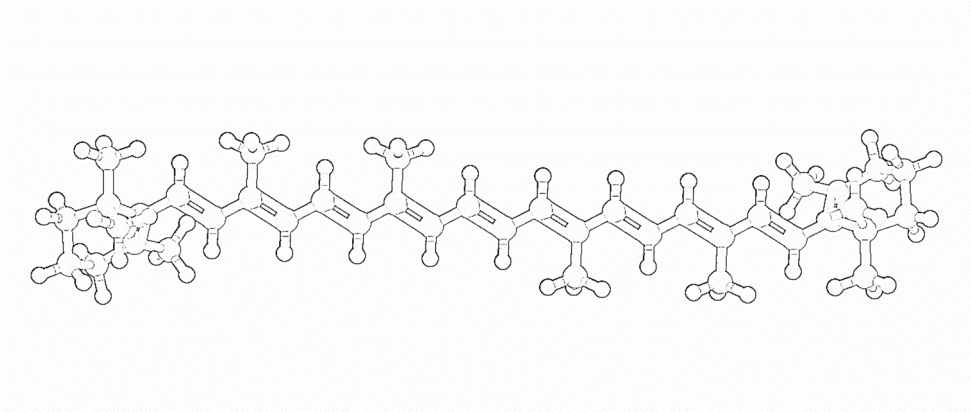
Figure 4. *Slaughtered equines (number of heads) in Spain in the last ten years*

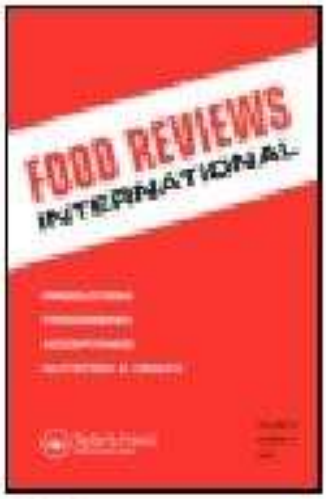
Graphics made from FAO (2014) data

For all these reasons, efforts to improve the consumers' awareness about the high nutritional value of these products are being made. The study of health-promoting compounds, such as carotenoids and fat-soluble vitamins, could help increase the acceptability of horse products in order to improve their consumption in countries like Spain, Italy, France and Belgium, where it remains low (Lorenzo et al., 2014).

Figure 5. *Hispano-Bretón breed animals*

INTRODUCTION





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Carotenoid and vitamin A content in biological fluids and tissues of animals as an effect of the diet. A review

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Carotenoid and vitamin A content in biological fluids and tissues of animals as an effect of the diet. A review

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ABSTRACT

Carotenoids are widespread isoprenoid secondary metabolites. They and their metabolites are thought to provide diverse health benefits. In any case, their relevance from a nutritional standpoint is unarguable as some of them can be converted into vitamin A in animals. Animals cannot synthesize carotenoids *de novo* and rely on the diet as a source.. Species-specific differences in the absorption and metabolism of these compounds are known to exist among livestock species. The diet is another key factor affecting the carotenoid content in biological fluids (like plasma and milk) and tissues. The study of carotenoids in animals is also important in the context of the increasing interest of consumers in the authentication of animal diets, as they can be used as markers of pasture-fed animals. In this paper we review the current knowledge on the effect of the diet on the carotenoid levels of domesticated animals and the differences in the metabolism of these compounds into vitamin A among species.

Keywords: animals, carotenoids; mammals; retinol; ruminants; carotenoid cleavage oxygenases; vitamin A; traceability; poultry

INTRODUCTION: IMPORTANCE OF THE STUDY OF THE PRESENCE OF CAROTENOIDS AND VITAMIN A IN ANIMALS

Carotenoids are isoprenoid compounds biosynthesized by photosynthetic organisms as well as some non-photosynthetic bacteria and fungi. They are characterized by a linear polyisoprene structure with conjugated double bonds and can be divided into cyclic or acyclic carotenoids and carotenes (hydrocarbons) or xanthophylls (carotenoids containing oxygenated functions) (1). They and/or their metabolites are involved in numerous actions/functions (2) and continue attracting an increasing interest due to a large body of evidence indicating that they can provide diverse health benefits (3,4). One of the main roles of carotenoids in mammals is as precursors of vitamin A (retinol $C_{20}H_{30}O$) (5). This vitamin is essential for cell growth and differentiation, reproduction, maintenance of immune system and vision, among other processes (6). Animals cannot synthesize it *de novo*, although they can form it from provitamin-A carotenoids. These possess, at least, an unsubstituted β-ring in their structure as well as an appropriate unsaturated backbone (such as β-carotene, α-carotene and β-cryptoxanthin) (7).

On the other hand, there is an increasing interest of consumers in healthy and nutritious foods with additional health promoting actions (8). In this sense, consumers are increasingly focusing both on the "green-image" as on the health properties of products from pasture-fed animals fed (9–12). Thus, research on diet authentication in herbivore products is conducted within a general

context of increasing consumer concern regarding the animal production system (9). Efforts have recently been made to develop analytical tools to quantify specific compounds in animal tissues that can act as tracers of the conditions of production (13). These compounds are transferred from the feed to the final product or transformed by the animal metabolism in response to specific diets. It is reasonable to think that they can be used to some extent to authenticate the diet of the animals and, in this way, help to reassure consumers as they can know where their food comes from and how it is produced (12,14). Carotenoids are examples of such compounds and they could be useful to differentiate pasture-fed from concentrate fed-animals (14–18).

In this context, this review summarizes current knowledge on the effect of the diet on the carotenoids and retinoids levels in different biological fluids and tissues from various animal species, as well as the differences in the metabolism of carotenoids into vitamin A among animal species.

ANIMAL CAROTENOID OXYGENASES: ENZYMES INVOLVED IN THE CLEAVAGE OF CAROTENOIDS

Carotenoids can be enzymatically cleaved into a series of metabolites like apocarotenoids and retinoids, among others. These are involved in important actions in nature. Retinoids are C20 compounds that can act as important signaling molecules and chromophores of photopigments (19). Most animals can metabolize carotenoids to diterpenoid molecules such as retinaldehyde, which can be subsequently converted into retinol or retinoic acid (19). Apocarotenoids are carotenoids with less than 40 carbon atoms, which are attracting increasing attention

(7,19,20,21). The cleavage of carotenoids into retinoids or apocarotenoids is catalyzed by carotenoid cleavage oxygenases (CCOs). For instance, mammalian genomes are known to encode at least two of these enzymes. Both CCOs depend on ferrous iron as a cofactor that binds to four conserved histidine residues in the active center, but do not contain a heme ring, so they belong to the family of non-heme iron oxygenases. β,β-carotene 15,15'–monooxygenase 1 (CCO1) cleaves centrally β-carotene into two molecules of all-*trans*-retinal, which is oxidized irreversibly into retinoic acid by retinal dehydrogenase or reduced reversibly into retinol by a retinal reductase. CCO1 is considered the key enzyme for β-carotene conversion into vitamin A. The other carotenoid cleavage enzyme, β,β-carotene 9',10'-dioxygenase (CCO2), cleaves β-carotene at the 9',10' double bond, forming β-apo-10'-carotenal and β-ionone (Figure 1). (7,19). The latter enzyme is thought to be also used for the synthesis of vitamin A (20).

CCO1 requires at least one unsubstituted β-ionone ring for the cleavage of carotenoids substrates, thus its activity is limited mainly to α- and β-carotene, β-cryptoxanthin and some β-apo-carotenals. On the other hand, CCO2 exhibits broader substrate specificity. It can also catalyze the excentric cleavage of non-provitamin A carotenoids at both the 9,10 and 9',10' double bonds producing both non-volatile apocarotenoid and volatile compounds cleavage products. CCO1 and CCO2 are localized in different intracellular compartments, CCO1 localizes to the cytoplasm, whereas CCO2 is a mitochondrial protein. This different localization suggests that carotenoid metabolism is compartmentalized, so that β-carotene is metabolized in the cytoplasm by CCO1, while other carotenoids are metabolized by CCO2 in the mitochondria. This compartmentalization has been suggested to prevent the competition of the two CCOs for β-carotene, which can be a limited nutrient in natural environments (7,20,21).

In mammals, CCO1 is expressed in the mucosal and glandular cells of the stomach, small intestine and colon, hepatocytes and cells in pancreas, prostate, endometrium, mammary tissue, kidney, skin, skeletal muscle, testis, ovary and adrenal gland. It is also found in the retinal pigment epithelium and in the ciliary body pigment epithelia of the eye. On the other hand, CCO2 can be found in almost all cell types that are known to express CCO1 but at lower levels. Moreover, the expression of both CCEs in the liver is different to other tissues, since both enzymes occur in separate hepatic cells type (7). In hepatocytes, which are parenchymal cells involved in uptake and processing of retinol, high levels of CCO2 can be found; while in stellate cells or hepatic fat-storing cells, which play a fundamental role in hepatic retinoid storage, high CCO1 levels are present (7).

Summarizing, two different carotenoid metabolizing enzymes are known. CCO1 is a cytosolic enzyme with narrow substrate specificity for pro-vitamin A carotenoids and CCO2 is a mitochondrial enzyme with a broader substrate specificity (19).

CAROTENOID CONTENT OF FEEDSTUFFS USED IN LIVESTOCK RATIONS

The main systems practiced for livestock production are the extensive (grazing) and the intensive (indoor) farming, although there are also other intermediate options. In the extensive grazing systems, animals are continuously stocked on a permanent pasture with no fed concentrate offered to them. Whereas, in the intensive indoor system, animals are housed indoors receiving a diet based on concentrates (22).

Forages

Forage refers to the vegetative portions of plants and includes natural or sown grassland (pastures), rough grazing, silage, hay, straw and annual and perennial green fodder from arable land. The carotenoid content is usually more abundant in fresh pasture since such content usually decreases following harvesting and preservation (25). Degradation of carotenoids occurs rapidly by oxidation, which can be accelerated by light exposure. Losses of carotenoids in forages can also be favored by several factors, like feed fertilization practices, filling of the silo, wilting, fermentation and/or storage. The carotenoid profile of green plant tissues is, with very few exceptions, very constant. The main carotenoids are invariably β-carotene and the xanthophylls lutein, violaxanthin, and neoxanthin. In addition, much lower levels of zeaxanthin, antheraxanthin and β-cryptoxanthin, among others, can be found. (Figure 2). Differences in the carotenoid content among botanical species have been described (23). It is also usual to find dramatic variations within the same species due to several factors, e.g.climatic, agronomic, genotypal. For example, lutein and β-carotene concentrations in green forages are two- to three-fold higher for the same species in humid versus dry tropics have been reported (1,24).

Concentrate feedstuff

Concentrate feedstuff represents a more concentrated source of energy and protein than forage. This diet is usually associated to animals indoors and, in the case of ruminants, it can be used to support a ration based on grass. There are concentrates based on wholegrain cereals and formulated concentrates containing specified proportions of individual ingredients, each having

specific nutritional roles (25). It has been pointed out that it is difficult to find reliable composition data concerning carotenoids for animal feeds (24), but some information is available. Thus, the predominant pigments in maize are lutein and zeaxanthin, with lower amounts of other xanthophylls, such as beta-cryptoxanthin, which are concentrated in corn gluten meal. Oxidation of carotenoids may occur during the manufacture of concentrate rations, as the production methods often involve heating, which can accelerate their degradation (24,25).

Industrial by-products used in animal feeding

The greatest constraint to livestock productivity is the shortage of forages and feed sources. The feeding system must be carefully chosen and controlled by the farmer due to its large contribution to the total production costs, which is one of the farmer's major concern (26,27). Agro-industrial by-products are of interest for the feeding of livestock, as they are less costly and could contribute to decrease the feeding system costs (27). In this sense, the use of vegetable by-products from greenhouses as a low-cost supplemental source of nutrition for livestock has been proposed (28). An example of these products is the pulp of citrus fruits, like oranges. The characteristic orange color of both the peel and the pulp of most varieties of ripe oranges is due the presence of several carotenoids (violaxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, zeinoxanthin, α-carotene, β-carotene, ζ-carotene), the profile becoming more intricate after industrial processing and storage, that can promote 5,6-epoxide to 5,8-furanoid re-arrangements and other isomerizations (29, 30). Additionally, output products from the industrial processing of tomato, which results in a range of products with different lycopene levels, can also be used for livestock feed production (31, 32).

RUMINANTS

Carotenoids can be important in relation to the sensory and nutritional quality of dairy products, whose composition is strongly influenced by the animals diet (33,34). The effect of this on the presence of carotenoids and vitamin A in fluids, tissues and products has been widely evaluated in cattle (13,17,34–38) and sheep (9,14–16,18,39–42), and to a lesser extend in goats (26,43–46). Despite the three species are ruminant, it is well-known that there are differences in the metabolism and accumulation of both carotenoids and retinoids (5,46).

Metabolism

According to Schweigert (5), mammals can be divided into two groups, namely "white-fat" and "yellow-fat" animals, considering the accumulation of carotenoids in adipose tissue. The first group is comprised of species that do not absorb carotenoids at all or absorb them at very low levels. Animals in the second group are those that do absorb carotenoids efficiently. In the case of ruminants, cattle belongs to the second group and small ruminants (goats, sheep) to the first (5). It has also been stated that the body fat colour of sheep and goats remains irrespective of diet, whereas that of cattle can vary from creamy-white to bright orange-yellow depending upon the diet (46). Yang *et al.* (46) suggested that these three ruminant species have different mechanisms involved in carotenoid absorption, deposition and metabolism, since fat colour is thought to result mainly from the accumulation of dietary carotenoids. In this sense, β-carotene has been reported (35,37,46) as the main carotenoid in serum and adipose tissue in cattle. Contrastingly, lutein has been reported to be the major carotenoid in sheep and its presence,

albeit at trace levels, has also been reported in serum and adipose tissue of goats (46). Interestingly, there are studies reporting lower duodenal and jejunal CCO1 activity in cattle compared with goats (47), as well as the more efficient intestinal CCO1-mediated cleavage of β-carotene to retinol in sheep than in cattle (39). In fact, the absorbed β-carotene is assumed to be almost entirely transformed into retinol in sheep. This is not the situation in the case of cattle, hence its appearance as the main carotenoid in plasma and some tissues. In sheep, β-carotene has been detected just at trace levels (39). It has been stated that cattle plasma β-carotene is mainly transported in High Density Lipoproteins (HDL) (48), which is thought to be the major (over 80%) lipoprotein fraction in plasma of ruminants, followed by Low Density Lipoprotein (LDL) (12.8-16.6%) and Very Low Density Lipoprotein (VLDL) (0.9-2.3%) (46). Interestingly, Yang *et al.* (46) established that carotenoids in bovine plasma are distributed approximately proportionally in VLDL, LDL and HDL, although in small ruminants they are mainly found in VLDL plus LDL (67.3% in sheep and 57.7% in goats) despite the much higher proportion of HDL. These authors also compared the liver retinol levels among the three species and concluded that it was significantly higher in sheep, although no differences were found for this compound neither in serum or adipose tissue among the three species (46).

Plasma

The carotenoid and retinol concentration in ruminants plasma reported in the literature vary greatly among studies (Table 1). β-Carotene only appeared in plasma of bovines (33–35,46), supporting the hypothesis regarding the lower CCO1 activity in cattle. Besides, most of the studies carried out in sheep expressed the carotenoids in plasma as plasma carotenoid

188 concentration (PCC), assuming that the only circulating carotenoid in these animals is lutein
189 (9,11,14,17,18,41). Finally, few studies have been carried out on the carotenoid profile in goat
190 plasma Yang *et al.* (46).

191 Besides lutein and β-carotene, other minor carotenoids have been reported in cattle plasma. For
192 instance, a geometrical isomer of β-carotene tentatively identified as (13*Z*)-β-carotene has been
193 detected (34,35) in cattle plasma fed with a diet based on grass plus concentrate and forage,
194 respectively. In this sense, Calderón *et al.* (34) stated that the exact site of β-carotene
195 isomerization remains unknown, although the rumen, enterocytes, liver, plasma or peripheral
196 tissues, including adipose tissue and mammary gland are all good candidates. In addition,
197 Calderón *et al.* (34) reported for the first time zeaxanthin in plasma (2.7 % of total plasma
198 carotenoids detected) although they did not detect it in milk.

199 On the other hand, the effect of the diet on the plasma carotenoid levels has been widely studied
200 (9,11,14,17,41). All these studies concluded that such levels depend on the diet (Table 1). It has
201 also been concluded that the concentrations increase linearly with the mean daily carotenoid
202 intake (14). Prache *et al.* (11) compared the persistence of carotenoids in plasma of lambs with
203 four feeding treatments: grass-feeding, stall-feeding, grass-feeding followed by a short stall-
204 finishing period, and grass-feeding followed by a long stall-finishing period. The authors
205 demonstrated that the overall persistence of carotenoids in the blood is low since after 4 to 13
206 days on the stall diet, plasma carotenoid content of stall-finished lambs decreased to the values of
207 the stall group. In terms of traceability, these results indicated that the stall-finished grazing
208 lambs may be considered as grass-fed during the first 4 to 13 days of the stall-finishing period

209 and as stall-fed thereafter. Moreover, this study suggests that the PCC depends to a large extent
210 to the finishing diet previous to slaughter of the animals.

211 Milk

212 Table 1 also summarizes data from the literature relative to the carotenoid and retinol milk
213 contents in the three ruminant species. It can be observed that varying the diet fed to livestock is
214 an efficient way of modulating carotenoids and vitamin A in milk (35). Thus, diets based on
215 pasture led to higher carotenoid and retinol levels in milk (33,43).

216 Furthermore, it is well known that the carotenoid pattern in milk is species dependent (49). In
217 this sense, Gentili *et al*. (50) reported in their study that milk from small ruminants was very rich
218 in vitamin A, but bovine milk was the only one containing appreciable levels of carotenoids
219 (Table 1). In addition, previous authors (43,45,51) reported retinol but no carotenoids in goat
220 milk. These observation can be explained by the already referred higher enzymatic conversion of
221 β-carotene into retinol in goat and sheep compared with cow (45,50). On the other hand,
222 Calderón *et al*. (34) reported that for cattle, when plasma β-carotene concentration exceeds
223 5μg/mL there is a limitation of the mechanism involved in the transfer of this carotene from
224 plasma to milk. This limitation of secretion may not be due to a higher cleavage of β-carotene in
225 the mammary gland, but rather to a limited uptake by the mammary gland or limited transport by
226 binding β-lactoglobulin and/or to saturation of milk fat globules, where carotenoids are found.
227 These facts, together with the lower CCO1 activity in cattle explained above, may explain the
228 presence of β-carotene in cow milk but not in sheep and goat milk, and the consequent yellower
229 colour of cow milk.

Adipose tissue

The study of Yang *et al.* (46) confirmed that, while cattle absorbs both lutein and β-carotene, lutein was the only carotenoid in adipose tissue of sheep and goats, although the amount of the latter was negligible compared with the carotenoids present in cattle (Table 2). Thus, the white colour of the body fat of sheep and goats is thought to be mainly due to the extremely low concentration of carotenoids in these animals Yang *et al.* (46).

Furthermore, the colour of bovine subcutaneous adipose tissue, i.e. carcass fat, is an important component on beef carcass quality and, thus, beef carcass grading (25). Yellow fat is positively associated with traditional grass-based beef production and is perceived as a positive quality criterion which is more "ecologically favorable" and may influence consumer purchase decisions (25). In this regard, adipose tissue from pasture-fed cattle has been shown (36,37) to have higher levels of β-carotene and lutein that than from concentrate-fed animals (Table 2). Besides, subcutaneous adipose tissue has been proposed (37) as a storage depot for β-carotene and lutein in cattle, which can be mobilized when the dietary supply of carotenoids is limiting. Moreover, Prache *et al.* (15) demonstrated that the rate of reduction in carotenoid concentration in the fat is much lower than in the blood, which implies that the assessment of carotenoid levels in different tissues can be a useful tool for feeding traceability purposes (52).

Liver

Liver is the primary storage site for vitamin A in mammals. In this context it is important to bear in mind that a ruminal degradation of dietary vitamin A has been reported in ruminants and that

degradation has been shown to take place in a diet dependent manner, being higher for concentrate than for forage diet (53).

Yang *et al*. (46) highlighted that, although lutein was the only carotenoid detected on serum and adipose tissue of sheep and goats (Tables 1 and 2), it was not detectable in their livers (Table 2). Contrastingly, β-carotene was present in their liver (Table 2) despite its absence from other sites, indicating that sheep and goats seem to have developed a mechanism that selectively deposits β-carotene in the liver where it can be converted to retinol when required Yang *et al*. (46). In products of animal origin vitamin A is mainly found as retinol and retinyl esters (54). Retinyl esters profile in liver of small ruminants has been, to our knowledge, poorly studied. Such profile is related to the typical bacterial modification of dietary fat in the rumen, where the free released unsaturated fatty acids are hydrogenated to saturated fatty acids (54). In this sense, retinyl palmitate (Figure 3) and stearate were predominant (54% and 32% of total vitamin A) in the livers of cattle.

MONOGASTRIC ANIMALS

In contrast with ruminants, there are few reports on the presence of carotenoids and retinoids in monogastric livestock. Within monogastric livestock animals important differences in the efficiency of carotenoid absorption between species have been reported. For instance, the absorption of carotenoids in pigs has been reported as extremely low, whereas carotenoids can be readily detected in the plasma of other species (reviewed in (5)), indicating differences in the different processes involved in the bioavailability of carotenoids. However, the underlying

reasons of the characteristic species-specific differences in carotenoids absorption and metabolism are not well understood yet. They could include differences in the absorption of carotenoids, the extent to which certain carotenoids are converted into vitamin A in the gut, or limitations to the secretion of carotenoids into the lymphatic circulation (reviewed in (5).

Metabolism

Horse is known (55) to absorb, circulate, accumulate and secret in milk the carotenoid β-carotene. The efficiency of the conversion of provitamin carotenoid in vitamin A in grazing horses has been shown to be relatively poor Greiwe-Crandell *et al.* (56). In addition, a homeostatic regulation of vitamin A in blood in mares similar to that reported in cattle (57) could be hypothesized according to preliminary results from our laboratory (unpublished data). This hypothesis could be supported by previous data indicating that β-carotene supplementation did not increase vitamin A concentration neither in mares nor in foal (58). Greiwe-Crandell *et al.* (56) established that β-carotene supplementation did not improve liver stores of vitamin A in mares, which adds support to the hypothesis about such homeostatic regulation. The mechanisms of this hypothetical regulation, as well as the general metabolism of carotenoids in horse, are not yet well understood. In any case, improving the knowledge on the accumulation of both carotenoids and fat-soluble vitamins in horse tissues would be interesting since, nowadays, efforts in extending the knowledge on equine products by improving consumer awareness of the high quality value of these products are being carried out (59). The study of the occurrence of health-promoting compounds like carotenoids could help increase the acceptability of horse products by the consumer (59).

On the other hand, pigs are known not to absorb intact dietary carotenoids efficiently (5), although detectable levels of retinol are found in bloodstream and tissues like fat and liver in Iberian pigs (60). The reasons why pigs do not absorb and accumulate carotenoids are not well understood yet. The study of carotenoids and retinoids in pig tissues appears interesting for several reasons. Firstly, it has been established (61) that an important determinant of the nutritional quality of pork meat, apart from its nutrient content, is the presence of bioactive compounds that can be beneficial for the health and well-being of consumers. In addition, Álvarez et al. (60) proposed retinoids levels in liver as biomarkers of feeding traceability in Iberian pigs, in order to differentiate *Montanera* (a typical outdoor rearing system with a nutritional strategy based on pasture and acorns) and *Cebo* (intensive system with a diet based on concentrate) feeding systems, since the highest Iberian pig quality products are related to *Montanera* feeding system.

Plasma

β-carotene has been reported as the only carotenoid present at detectable levels in horse plasma (Table 3). Dietary intake has been described (62) as the major determinant of β-carotene concentration in mare plasma. Kuhl et al. (58) stated that an oral supplementation with β-carotene increased its concentration in plasma of mares but there was further increase over time, independent from oral β-carotene supplementation. When the effect of different diets on the plasma carotenoid concentration in mares was studied (56), no differences were found in β-carotene level among the three diets studied (Table 3). Additionally, it was reported that in almost all of the mares receiving a diet of concentrate plus hay the β-carotene concentration in

312 serum was below detectable limits. However, the range of serum carotenoid levels in the mares
313 in which detectable serum levels of β-carotene were found was in accordance with other studies
314 (Table 3). No intact carotenoids have been reported in plasma in pigs independently of the diet
315 received (Table 3).

316 Both horses and pigs, have been reported to circulate retinol in plasma (Table 3). No differences
317 (Table 3) in plasma retinol level as a function of the diet have been reported neither in horse nor
318 in pig (56,58,60). Thus, Kuhl et al. (58) reported that the concentration of retinol in plasma of
319 mares and foals was constant throughout the observation period. These data would agree with the
320 hypothesis of a homeostatic regulation of retinol in horse referred to above.

321 Adipose tissue

322 There are few studies on the content of carotenoids and retinoids in the adipose tissue of
323 monogastric livestock. Olivares et al. (63) compared retinol subcutaneous back fat content in
324 pigs with a control diet and a vitamin A enriched diet, concluding that the concentration of such
325 vitamin was higher ($P<0.001$) in the pigs that were receiving the vitamin A enriched diet. In
326 addition, Álvarez et al. (60) studied the effect of two different carotenoid-containing diets
327 (pasture + acorns *vs.* concentrate) on the retinol levels in perirenal fat of Iberian pigs, concluding
328 that they were not significantly different.

329 On the other hand, there is little information on the carotenoid and retinol concentration in horse
330 adipose tissue. Despite horses having been shown to absorb carotenoids, these were not found in
331 the perirenal fat of foals, although retinol was (unpublished data from our laboratory). However,

this fact could be due to the low amount of carotenoids present in the diet of the animals studied, since they received a diet based on concentrate (that is poor in these compounds) without vitamin A supplementation.

Liver

It has been reported that, in mammals, up to 80% of the body's total retinol is present in the liver, mainly located in stellate cells. Retinol esterified with various long-chain fatty acids (retinyl esters) is the major storage form (64). Data on liver retinol levels have been reported (Table 3). Significant differences have been found (60) in the liver of Iberian pigs depending on their diets, which can be useful to discriminate Iberian pigs according to their diet in the context of feeding traceability. Retinyl esters profile in pig liver had also been studied previously (54,60). The composition of retinyl esters in pig liver has been reported to reflect the fatty acid profile of a typical diet for omnivores (54). In this way, retinyl palmitate has been reported in both studies as the predominant form of vitamin A. Besides, retinyl palmitate and all-*trans*-retinol, the vitamin A fraction in pig liver is composed by retinyl oleate, retinyl stearate and retinyl linoleate (54,60). An effect of the diet on the retinoid profile in Iberian pig liver has been demonstrated by Álvarez et al. (60), so that they were able to discriminate the 92.9% of the animals according to their diet (pasture + acorns *vs.* concentrate) considering the concentration of all-*t*-retinol and retinyl esters in liver samples.

To the best of our knowledge, there are no published data on the retinyl esters profile in horse liver. Nevertheless, we have observed that foals fed on concentrate and slaughtered when they were around 14 months old accumulate all-*trans*-retinol, retinyl linolenate, retinyl linoleate,

353 retinyl oleate, retinyl palmitate and retinyl stearate in their livers (unpublished data). Retinyl
354 palmitate was found to be the predominant form, which agrees well with the results of Majchrzak
355 et al. (54), who stated that this ester is the main form of vitamin A in the liver of different
356 species.

357 Milk

358 Although mare's milk production does not have economic importance and long traditions as
359 production of milk from ruminants, it is gaining importance in European countries. This is due in
360 part to its interesting composition, such that mare's milk can be a profitable alternative to cow's
361 milk especially among people allergic to the latter (65). The nutritional quality of dairy products
362 is highly correlated with milk fat quality and therefore with high fat-soluble vitamin contents
363 (65). Some studies on the content of β-carotene and retinol in mare milk have been carried out in
364 the last years (Table 3). Mares absorb dietary β-carotene, and this is found in plasma, tissues and
365 milk (55,58,62,65) (Table 3). Retinol is also found in mare milk (Table 4). According to
366 Schweigert & Gottwald (55), β-carotene levels in mare milk depend on its circulating levels,
367 albeit retinol presence in milk seems to be independent of its level in plasma. They hypothesized
368 that this fact would imply a more passive transfer of β-carotene compared to vitamin A into mare
369 milk. Finally, it is also thought that, in general, the concentrations of β-carotene and fat-soluble
370 vitamins in mare milk are comparable to those found in cattle, so mare milk can be a valuable
371 source of these components in the human diet (65).

372

POULTRY AND OTHER BIRDS

Carotenoids pigments provide color to the integument of many birds' species, being responsible for many of their red, orange and yellow hues. Since birds cannot synthesize carotenoids *de novo,* they must acquire them directly from the diet (66).

Metabolism

Mammals are known to absorb β-carotene better than birds, but among birds there are some species, such as goose, that exhibit a particularly good ability to absorb some carotenoids. For example, zeaxanthin has been reported to be absorbed three times better than astaxanthin under certain conditions (67).

In birds, as in mammals, provitamin A carotenoids are converted into retinol by CCO1, mainly in the intestinal mucosa. The retinol formed is stored in the liver and abdominal fat of the animals, mainly as retinyl palmitate. It has to be taken into consideration that, despite the mechanism of conversion is the same than in mammals, in birds the efficiency of such conversion has been shown to decrease with high intakes of β-carotene (67). Finally, many species of birds metabolize dietary carotenoids into more oxidized forms that are deposited into the integument, but the enzymes responsible for these carotenoids conversions have not been characterized so far (68).

Plasma

Some studies have been carried out on the content of carotenoids in plasma from different bird species (66,69–71). The variation in the carotenoid content and composition in the diet has been pointed out as the main reason for the differences in plasma carotenoid levels among populations of the same bird species (72). Lutein has been reported (70,71) as the major and most prevalent carotenoid in bird plasma. It also has been shown to be found to tissues like skin, liver and adipose tissue. Additionally, zeaxanthin, β-cryptoxanthin and cantanxanthin have also been found in the serum of various avian species. Contrastingly, the presence of β-carotene has not been detected (73). In fact, Jamroz et al. (67) confirmed the absence of β-carotene in serum of birds, specifically in goose plasma, even after β-carotene supplementation. Other xanthophylls like 3-hydroxy-echinenone have also been reported (66) in the circulation.

Adipose tissue

Birds are able to store carotenoids in different tissues, such as adipose tissue (74). Negro & Garrido-Fernández (71) reported the presence of lutein and one of its *cis*-isomers, β-carotene, β-cryptoxanthin, neochrome and neoxanthin in goose fat. Lutein and its *cis*-isomer were the major carotenoids, accounting for about 50% of the total carotenoid content. Surai et al. (73) studied the carotenoid profile in gull fat, describing the presence of cantanxanthin, β-carotene, β-cryptoxanthin and echinenone. As a result of these and other studies it can be readily inferred that these bird species are able to absorb and store in fat more carotenoids than mammals, so the

410 study of their different mechanisms of absorption and storage appears as an interesting research
411 field.

412 Liver

413 Carotenoids and retinoids composition in avian liver has been more widely studied than other
414 tissues because of the important commercial value of "foie gras" or "fatty liver" (75). Avian
415 species are very efficient converters of β-carotene to vitamin A and the liver is considered to be
416 the major organ of vitamin A accumulation in the form of retinyl esters (73). Retinyl palmitate is
417 known to be the main retinyl form in liver of gulls (59.6% of vitamin A content in liver) (73),
418 chicken (40.0%) and turkey (36.0%) (54). Other retinyl esters have been described in bird livers
419 (gull, chicken and turkey), such as retinyl oleate, retinyl stearate and retinyl linoleate. Retinyl
420 stearate levels appear to be higher than those of retinyl oleate and retinyl linoleate (54,73).

421 Carotenoids have also been described in bird livers. According to Surai et al. (73), gull tissues
422 are characterized by comparatively high carotenoid concentrations with the liver having the
423 highest. In their study, the liver exhibited the highest level of β-carotene (51.2% of total hepatic
424 carotenoids), but lower proportions of lutein and zeaxanthin compared with other tissues. They
425 hypothesized the possible existence of lutein/zeaxanthin-binding protein in avian liver, so these
426 mixture of carotenoids would be absorbed in the intestine and delivered to it. After that, liver
427 carotenoids may be incorporated in newly synthesized VLDL that are responsible for their
428 delivery to peripheral tissues which would become enriched with these particular carotenoids.
429 Other carotenoids, mainly β-carotene, would be retained in the liver becoming this tissue

enriched in this carotenoid in comparison to peripheral tissues. Finally, these authors also reported other carotenoids in gull liver, such as cantanxanthin, β-cryptoxanthin and echinenone.

On the other hand, del Val et al. (66) reported the presence of 3-hydroxy-echinenone in crossbill liver hypothesizing that, since carotenoids are delivered to peripheral tissues through the bloodstream from sites of conversion and storage, the presence of 3-hydroxy-echinenone in liver and plasma of crossbills could be explained by a hepatic conversion of dietary β-cryptoxanthin and subsequent delivery of the resultant 3-hydroxy-echinenone to follicular cells via plasma. β-Cryptoxanthin and β-carotene have also been reported in bird liver, specifically in house finches liver (68).

ANIMAL TISSUES COLOUR AND CAROTENOIDS

Colour perception of food quality plays a major role in consumers' evaluation (76). Consumers seek first to be entirely satisfied with the sensory properties of products, before other quality dimensions become relevant. As it has been commented above, carotenoids contribute to the colour and nutritional quality of food products from herbivores and have been proposed as potential biomarkers for authenticating carcasses produced from animals fed on pasture systems, in sheep (9,40,77,78), cattle (17,37) and Iberian pigs (60). The relationship between the carotenoid concentration in adipose tissue of animal fed on pasture and its yellow colour has been evaluated (25). Prache & Theriez (40) proposed a mathematical analysis of the fat reflectance as an indirect system to estimate the concentration of carotenoids in order to discriminate the two main production systems (extensive *vs.* intensive) in herbivores. These

450 authors showed that the reflectance spectrum of caudal adipose tissue of grass-fed lambs can be
451 distinguished at slaughter from that of lambs fed concentrates by the presence of absorbance
452 shoulders between 450 and 510 nm. This is a region where carotenoids absorb light strongly. In
453 this way, they proposed a mathematical analysis to obtain translated reflectance values (TR_i) and
454 the Absolute Value of the Integral (AVI) of the translated spectra. To do this, the reflectance
455 spectra between 510 and 450 nm must be translated to make the reflectance value at 510 nm
456 equal to zero (TR) (Figure 4). Thus, the translated reflectance values (TR_i) have to been
457 calculated from the reflectance values (R_i) as follows: $TR_i=R_i-R_{510}$, with i= 360, 370, 380…740;
458 whereas AVI of the translated spectra were calculated according to the following formula;

459 $AVI= [(TR450/2) + (TR460 + TR470 + TR480 + TR490 + TR500 + TR510/2)] \times 10$

460 Following this mathematical study, previous authors (9,77) have demonstrated that animals fed
461 on pasture show lower absorbances in adipose tissue than those fed on concentrate throughout
462 the full visible spectrum. Besides, the usefulness of the translated fat spectrum to differentiate
463 lambs according to their feeding system (grazing *vs.* concentrate) has also been established (78).
464 Moreover, in previous studies (9,15,77) the absolute value of the integral (AVI) of the translated
465 spectra between 450 and 510 nm was proposed as a tool for feeding traceability in lambs, since
466 AVI values for grass-fed animals were confirmed as significantly higher due to the presence of
467 carotenoids in the adipose tissue, than for stall-fed lambs. Additionally, a measurement of the
468 reflectance spectrum of the fat shows obvious practical interest since it is a non-invasive
469 methodology, takes little time and can be easily implemented in the meat industry with a portable
470 spectrophotometer (9). Likewise, it has been established (15,17) a positive correlation between

plasma carotenoid concentration at slaughter and AVI. In relation to this, Prache et al. (15) proposed the combined use of both plasma carotenoid concentration at slaughter and AVI as a more precise assessment for the discrimination of lamb production system.

The differentiation of Iberian pigs according to their diet (pasture + acorns *vs.* concentrate) based on the TR_i and AVI analysis of perirenal fat was not successful (60). This fact was probably due to a great extent to the absence of carotenoids in pig fat. Thus, it was concluded that, in contrast to ruminant species which absorb carotenoids, neither the reflectance spectra not the AVI value seemed to be useful to differentiate pigs according to their diet (60).

CONCLUSIONS

Species-specific factors exist in absorption, metabolism and storage of carotenoids in livestock. The diet of the animals is an important factor that affects the content of these pigments in the biological fluids and tissues of the animals, although more studies in this regard are needed in some species, such as many birds. In addition, research on animal diet authentication is a challenge for scientific research since nowadays there is general consumer interest in the animal production system. In this sense, carotenoids and its relation with animal tissues colour may be useful in some cases, as it has been suggested in some studies with ruminants.

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718

719 **Table 1.** Effect of the diet on lutein, β-carotene and retinol in milk and plasma of dairy
720 ruminants.

Reference	Species	Diet	Lutein		β-carotene		Retinol	
			Milk	Plasma[1]	Milk	Plasma[1]	Milk	Plasma[1]
(46)	Sheep	Pasture		0.006 b				0.35
	Goat	Pasture		0.004 a		2.19		0.35
	Cow	Pasture		0.057 c				0.28
(79)	Cow	Concentrate				0.002		1.8
(11)[2]	Sheep	Grass		0.063a				
		Stall		0.012 b				
		Grass + short stall-finished		0.009 bc				
		Grass + long stall-finished		0.007 c				

(43)[3]	Goat	Pasture					650.5 a	
		Concentrate					498.6b	
(33)[4]	Cow	Corn silage + temporary grassland	0.37		4.4		7.6	
			0.46		4.4		7.3	
		Permanent grassland + temporary hay	0.49		3.8		6.3	
		Permanent grassland hay						
(34)[5]	Cow	Grass (hay + silage + alfalfa) + concentrate	0.024	0.50	0.10	4.40	0.18	0.44
(9)[2]	Sheep	Pasture		0.075 a				
		Concentrate		0.010 b				
(14)[2]	Sheep	Pasture		0.112				

		Concentrate + alfalfa		0.003				
(51)[5]	Goat	Pasture					0.13	
(17)[2]	Cow	Pasture					5.92 a	
		Concentrate (hay)					0.18 b	
(41)[2]	Sheep	Pasture		0.103				
		Dehydrated alfalfa (stall-fed)		0.079				
(35)[5]	Cow	Forage			0.22	1.85	0.19	0.67
(50)[5]	Cow	Pasture	0.01		0.24		2.1	
	Sheep		0.01				4.3	
	Goat						4.3	
(18)[2]	Sheep	Concentrate + alfalfa + barley		0.108-0.129				

721 Means in the same column and the same experiment with different letters (a, b and c) differ
722 significantly ($P<0.05$).

723 [1]µg/mL

724 [2]PCC: Estimation of total Plasma Carotenoid Concentration, considering that lutein is the only
725 carotenoid in serum of sheep.

726 [3]milk concentration expressed as µg/ 100 Dry Matter (DM)

727 [4] milk concentration expressed as µg/ g fat

728 [5] milk concentration expressed as µg/ mL milk

729

730 **Table 2.** Effect of the diet on lutein, β-carotene and retinol in fat and liver of ruminants.

Reference	Specie	Diet	Lutein		β-carotene		Retinol	
			Fat[1]	Liver[2]	Fat[1]	Liver[2]	Fat[1]	Liver[2]
(46)	Sheep	Pasture	0.02 b			0.087 b	0.92	48.4 a
	Goat	Pasture	0.01 b	0.03	0.81	0.069 b	1.18	13.6 b
	Cow	Pasture	0.17 a			0.701 a	0.99	14.3 b
(79)	Cattle	Concentrate				0.08	1.0	10.1
(80)	Cattle	Pasture			0.99	1.21		
		Grain			0.10	0.08		
(15)	Sheep	Grass	0.025					
		Stall	0.009					
		Grass + short stall-finished	0.006					
			0.010					
		Grass + long stall-						

		finished				
(36)	Cattle	Pasture		0.28 a	0.37	
		Concentrate		0.18 b	0.22	
(54)	Cattle	Concentrate	+			4.10
		pasture				2.01
		Cow's milk				
(37)	Cattle	Pasture		0.14 a	0.55 a	
		Concentrate		0.04 b	0.10 b	

731 Means in the same column and the same experiment with different letters (a, b and c) differ
732 significantly ($P<0.05$). [1]µg/ g [2] mg/100 g

733

734 **Table 3.** Effect of the diet β-carotene and retinol in plasma, milk and liver of monogastric
735 livestock.

Reference	Specie	Diet	β-carotene		Retinol		
			Plasma[1]	Milk[1]	Plasma[1]	Milk[1]	Liver[2]
(56)	Horse	Pasture + hay	0.21		0.22		
		Pasture + hay + concentrate	0.24		0.22		
		Hay + concentrate	0.17		0.20		
(55)	Horse	Hay + grass cobs + corn silage + barley	0.26-0.59	0.011-0.067	0.17-0.19	0.007-0.025	
(62)	Horse	Pasture + concentrate	0.36	<0.03	0.17-0.19	<0.25	
(54)	Pig	Concentrate					11.5
(58)	Horse	Concentrate	0-0.25	0.003 b	0.15-0.20	0.10-0.50	
		Concentrate + β-carotene	0-0.60	0.037 a	0.15-0.20	0.10-0.60	

		(1000 mg/day)			
(65)	Horse	Oat + hay + straw	0.388	0.403	
(60)	Pig	Pasture + acorn	1.07		37.0 a
		Concentrate	0.68		12.0 b

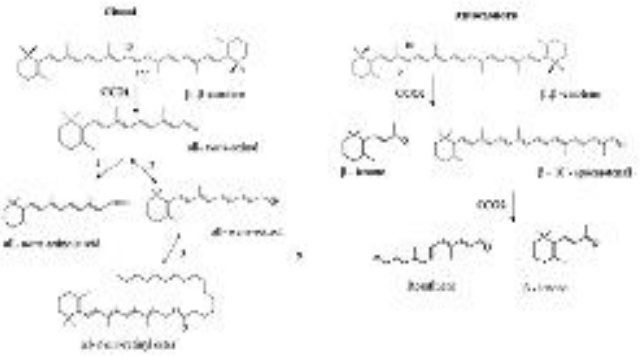
736 Means in the same column and the same experiment with different letters (a and b) differ
737 significantly ($P<0.05$).

738 [1] µg/mL

739 [2] mg/100 g

740

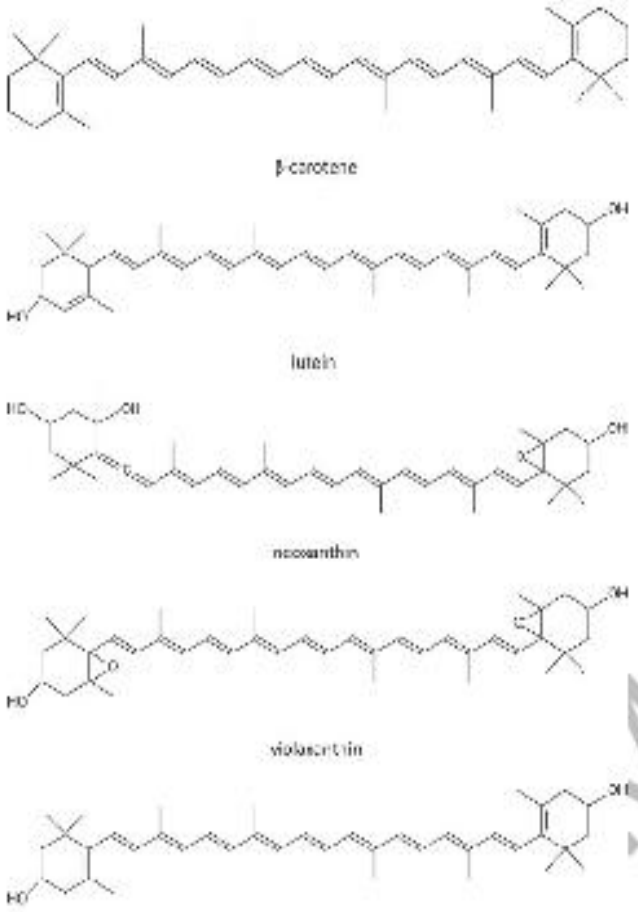
741 **Figure 1.** Schematic representation of β,β-carotene metabolism mediated by CCO1 (β,β-carotene
742 15,15'–monooxygenase 1) and CCO2 (β,β-carotene 9',10'-dioxygenase).



743

744

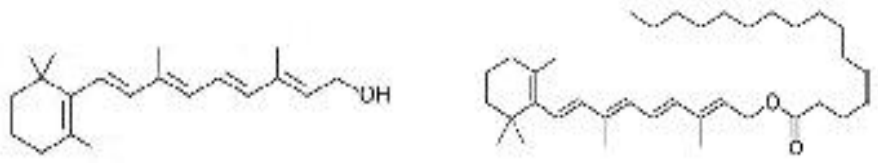
745 **Figure 2.** Carotenoids present in livestock feedstuffs (1, 23, 24).



746

747

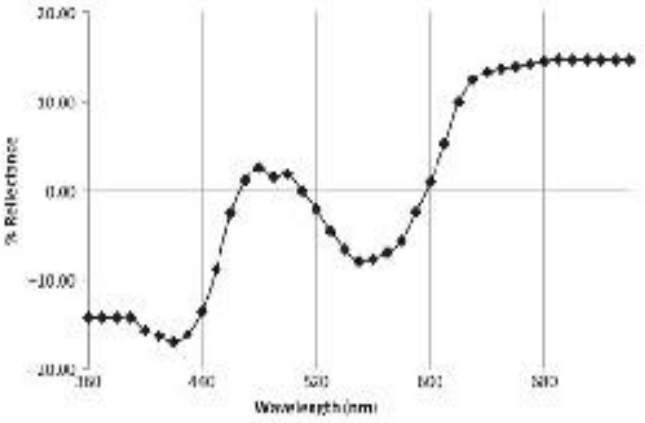
748 **Figure 3.** Main retinoid forms detected in mammal's liver (54).



749 All-trans-retinol Retinyl palmitate

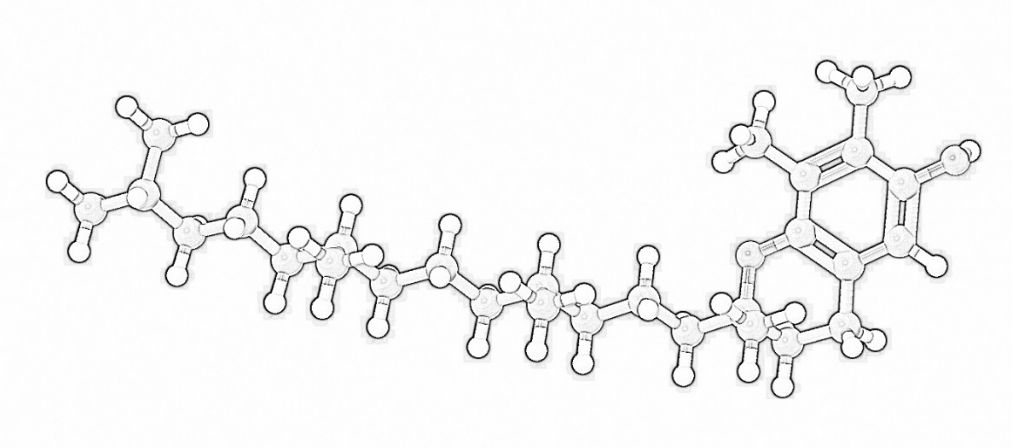
750

751 **Figure 4**. Averaged translated reflectance spectra (AVI) between 360 and 740 nm of perirenal
752 fat of Iberian pigs (60).



753

OBJECTIVES

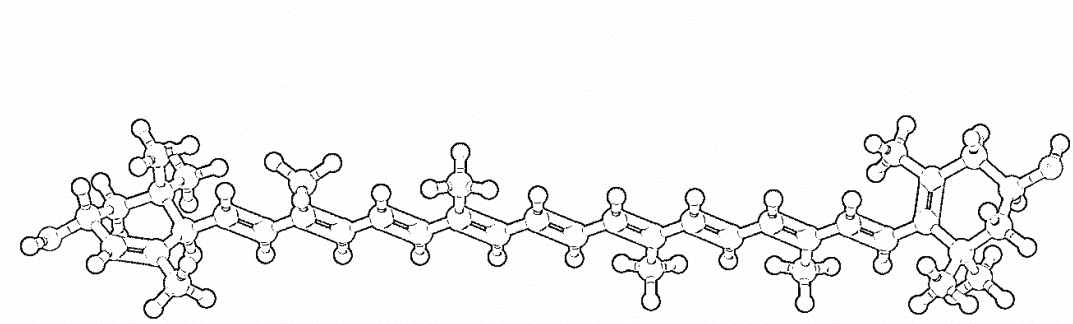


OBJECTIVES

Carotenoids, retinoids and tocopherols are important health-promoting compounds for humans. Their occurrence in animal tissues and products depends on the diet. In this sense, animals reared on extensive system with a diet based on pasture are better accepted by consumers that those reared on intensive system fed on concentrated diets. Also the extensive production system is considered more ethical and welfare-friendly with the animals. In this context, the main objective of this doctoral thesis was to gain an insight into the influence of the rearing system on the carotenoids and tocopherol composition of different animals tissues. Monogastric and ruminant animals were included in the study. The particular objectives were:

- To study the effect of different diets on the levels of carotenoid and retinol in goat´s milk and plasma.
- To analyze the effect of different diets on the carotenoids and fat-soluble vitamins (retinol and α-tocopherol) levels in sheep´s biological samples in relation to the production system conditions.
- To evaluate the usefulness of carotenoids, retinol and α-tocopherol levels in combination with colour measurements in sheep fat to differentiate stall-fed from pasture-fed lambs.
- To assess the effect of different carotenoid-containing diets on the vitamin A levels and colour parameters of Iberian pigs' tissues.
- To evaluate the utility of retinoids levels in different tissues as well as the perirenal fat colour to differentiate feeding systems in Iberian pigs.
- To assess the levels of carotenoids, retinoids and α-tocopherol in different horse biological fluids and tissues and to compare it with cattle.

MATERIAL AND METHODS



MATERIAL AND METHODS

Sampling methodologies

Feedstuffs

Representative samples of the diets of the different livestock species studied were collected in triplicate. In the case of pasture groups, the sampling areas were randomly selected at the prairie where the animals were reared and samples were collected in spring. The pasture was sampled from the ground using definite quadrates arbitrarily established. Finally, the pasture samples were overall characterized at the genus level considering the major herbaceous species present. They were transported to the laboratory, freeze-dried and stored at -80 ºC until analysis.

Animal tissues and biological fluids

Plasma

Blood from living animals (lactating goats, sheep, cows and mares) was taken from the jugular vein (goats, sheep and mares) and the caudal vein in the case of cows. Blood from sacrificed animals (lambs, pigs, foals and calves) was also taken at the moment of slaughter. Li-Heparin was used as anticoagulant in both cases. The samples were transported to the laboratory at 4 ºC, and centrifuged (1500 g, 10 min, 4 ºC). The plasma was collected and stored at -80 ºC until analysis.

Adipose tissue and liver

Samples of liver and perirenal fat were also taken from the animals that were slaughtered (Chapters 2, 3 and 4). Sampling was made at the moment of the slaughter, during the dressing of the carcasses. Perirenal area was selected for adipose tissue sampling as, according to Priolo et al. (2002), there may be a greater accumulation of

carotenoid pigments in perirenal compared to caudal fat in lambs. The samples were transported to the laboratory and stored at -80ºC until analysis.

Milk

Two aliquots of both morning and afternoon milking were mixed to obtain a representative sample of each animal. In total, 30 mL of milk from each animal were aseptically collected into sterile vials and immediately stored at -80ºC until analysis. The milk samples were collected the same week of lactation for the animals of the same experiment (goats in chapter 1, sheep in chapter 2 and cows and mares in chapter 4).

Carotenoids and tocopherols extraction in feedstuffs

A good extraction procedure should recover all the analites of interest in solution, without any change in them (Mínguez-Mosquera, 1997). As carotenoids and tocopherols are very unstable some precautions were taken during analysis in order to avoid their oxidation and loss (Meléndez-Martínez et al., 2004; Mínguez-Mosquera, 1997):

- Handling time of the extracts was minimized.
- Light exposure and heating were avoid in order to reduce the risk of isomerization and oxidation.
- Solvents free of impurities, mainly free of peroxides and acids were used.
- Oxygen was removed by displacement with nitrogen or working under vacuum atmosphere.
- Antioxidants were added to the solvents.

Carotenoids and tocopherols analysis in feedstuffs were carried out according to the methodology described previously (Kean et al., 2012; Pickworth et al., 2012) with some modifications. One gram (g) of sample (pasture, concentrate or other specific diet sample)

was freeze-dried. 3 milliliters (mL) of hexane/ethanol mixture (1:1, v/v) were added to the freeze-dried material and centrifuged at 3500 rpm and 4 ºC over 10 minutes (Allegra X-22R Centrifuge, Beckman Coulter, USA). The extraction was repeated until colour depletion of the samples. Organic phases were pooled and saponified with 15% (w/v) ethanolic potassium hydroxide solution overnight at room temperature in the dark and under nitrogen atmosphere. Finally, the organic phase was washed several times with water up to all the potassium hydroxide was removed. After that, the organic phase was collected and dried using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The dry residue was stored at -20 ºC until the moment of the HPLC analysis.

Carotenoids, retinoids and tocopherols extraction in animal biological fluids and tissues

Plasma

A methodology described elsewhere was used with some modifications (Lyan et al., 2001). 2 mL of plasma were deproteinized by adding 2 mL of ethanol. Carotenoids, retinol and tocopherol were extracted twice with 2 mL of hexane with centrifugation (3500 rpm, 10 min, 4ºC; Allegra X-22R Centrifuge, Beckman Coulter, USA). The extracts were pooled and evaporated to dryness with a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The dry residue was stored at -20 ºC until the moment of the HPLC analysis.

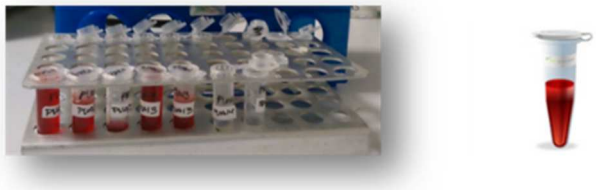


Figure 6. *Plasma samples*

Adipose tissue

The extraction was carried out following the methodology described by Dunne et al. (2006) with some modifications. 500 milligrams (mg) of perirenal fat were mixed with 1 mL of 3,5-di-ter-4-butylhydroxytoluene (BHT) ethanolic solution (12% w/v) in order to suppress the oxidation of the analites. 5 mL of ethanolic sodium hydroxide solution (30% w/v) and 5 mL of ethanol were also added. The saponification reaction was carried out overnight at room temperature and in the dark. The next morning water was added to stop saponification and the analytes were extracted with 10 mL of a mixture ether/hexane (2:1 v/v). Samples were centrifuged (3500 rpm, 10 min, 4 ºC; Allegra X-22R Centrifuge, Beckman Coulter, USA) and the upper organic phase collected. The extraction was repeated twice. The organic phases were pooled, washed several times with water, collected and dried (Concentrator plus, Eppendorf Research®, Madrid, Spain). The dry residue was stored at -20 ºC until the moment of the HPLC analysis.

Liver

In the case of liver samples, the methodology described by Woodall et al. (1996) was followed with some modifications. 0.1 g of liver was freeze-dried and the dried samples were homogenized in 500 µl of saline solution 85% (w/v). Then, compounds were extracted with 1 mL of dichloromethane by vortexing and centrifugation (3500 rpm, 10

min, 4 ºC; Allegra X-22R Centrifuge, Beckman Coulter, USA). Lower organic phase was collected and extraction was repeated until colour depletion of the samples. Saponification was not performed in order to identify and quantify retinyl esters with long-chain fatty acids in liver samples. The organic phases were pooled and dried (Concentrator plus, Eppendorf Research®, Madrid, Spain). The dry residue was stored at -20 ºC until the moment of the HPLC analysis.

Milk

The extraction of carotenoids, retinol and tocopherol from milk was carried out considering previous methodologies (Hulshof et al., 2006; Jewell et al., 2004; Nozière et al., 2006b). 1 mL of milk was mixed with 0.5 mL of water, 0.25 mL of ammonia aqueous solution 25% (v/v), 1 mL of ethanol and 1 mL of hexane. The mix was then centrifuged (3500 rpm, 10 min, 4ºC; Allegra X-22R Centrifuge, Beckman Coulter, USA) and the upper organic phase collected. The extraction was repeated until colour exhaustion. The organic phases were pooled and 2 mL of ethanolic sodium hydroxide 15% (w/v) was added. The air of the containers was replaced with nitrogen and the saponification reaction was maintained overnight at room temperature in the dark. Finally, the organic phase was washed several times with water, collected and dried (Concentrator plus, Eppendorf Research®, Madrid, Spain). The dry residue was stored at -20 ºC until the moment of the HPLC analysis.

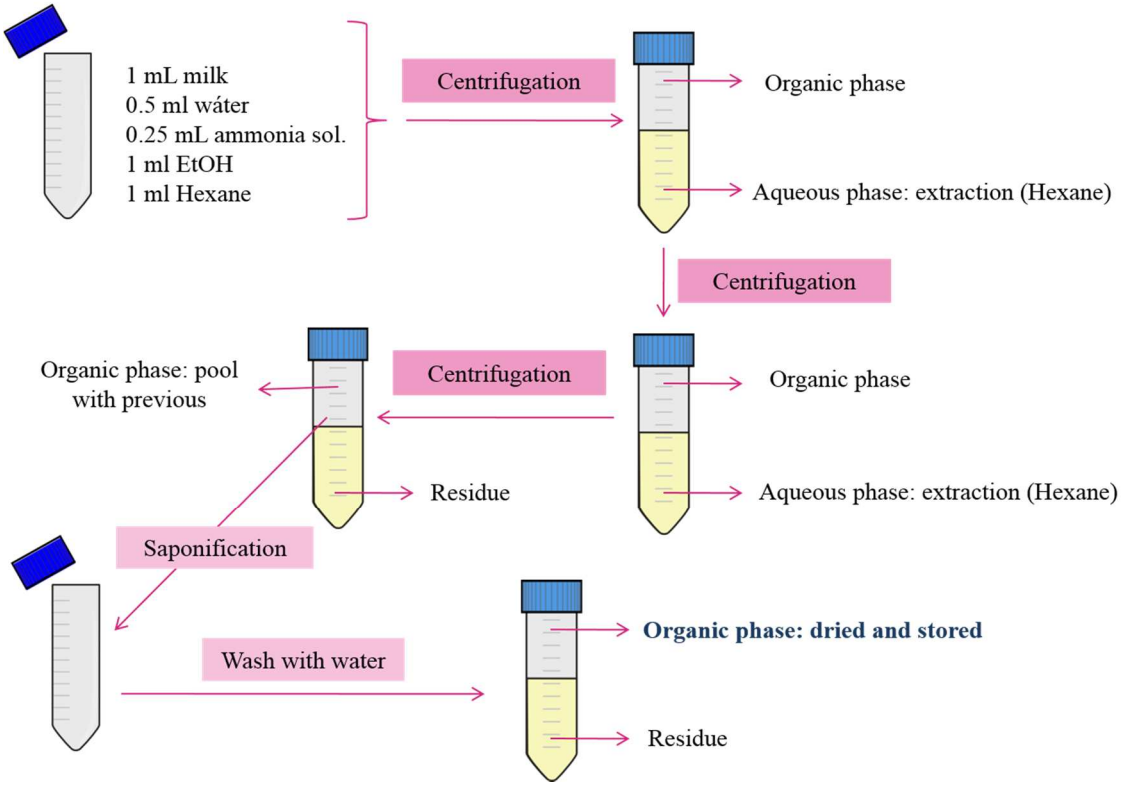


Figure 7. *Scheme of the methodology followed for carotenoid, retinol and tocopherol extraction in milk*

High Performance Liquid Chromatography (HPLC) analysis

The analyses were performed on an Agilent 1100 system (Agilent, Palo Alto, CA, United States) fitted with a photodiode array detector, a quaternary pump, a column temperature control module set at 20 ºC and an auto sampler set to draw 20 µl aliquots from the concentrate extracts. YMC C_{18} and YMC C_{30} columns were used (5µm, 250 x 4.6 mm) (YMC, Wilmington, NC, USA) were used for the analyses of feedstuffs and animals biological fluids and tissues. The mobile phase consisted on:

- Phase A: Methanol (MeOH) (Merck, Ref 1.06018).
- Phase B: Methyl-ter-butyl ether (MTBE) (Merck, Ref 1.01845)

- Phase C: Water MilliQ (filtration equipment NANOpure Dlamond, Barnsted Inc. Dubuque, IO).

The linear gradient used is summarized in table 1.

Table 1. *Gradient elution used in the HPLC determination of carotenoids, retinoids and tocopherol*

Time (min)	% A	% B	% C
0	90	5	5
12	95	5	0
25	89	11	0
40	75	25	0
50	40	60	0
56	15	85	0
62	90	5	5

The mobile phase was pumped at 1mL/min and the chromatograms were monitored at 450 nm for carotenoids, at 325 nm for retinoids and at 280 nm for tocopherol. Every day when the analyses were finished the column was washed with MTBE:MeOH (50:50) for 20 minutes.

Carotenoids identification and quantification

Carotenoids were identified by comparison of their spectroscopic and chromatographic characteristics with those of standards obtained from natural sources by recommended procedures as described elsewhere (Meléndez-Martínez et al., 2009). Table 2 shows the spectroscopic characteristics of the main carotenoids identified in this doctoral thesis.

Table 2. *Spectroscopic characteristics of main carotenoids identified in the samples analyzed in this study*

Carotenoid	Absorption maxima (nm)
β-carotene	452 472
β-cryptoxanthin	446 471
Lutein	424, 444, 472
Zeaxanthin	424 450, 476
Anteraxanthin	421 446 473
Violaxanthin	416, 440, 470

Carotenoids in solution follow the Beer-Lambert law, i.e. their absorbance is directly proportional to the concentration, thus their quantification can be performed by relating the spectrophotometry absorbance with a certain wavelength considering an absorption coefficient. The specific absorption coefficient (*A*) is defined as the theoretical absorbance of a solution with a concentration 1% (w/v) in a cuvette of 1 cm path length. Besides, the molar absorption coefficient (ε) is defined as the theoretical absorbance of a

solution 1 molar concentrated. Both coefficients are related according to the following formula:

$$\varepsilon = \frac{A \text{ x } MM}{10}$$

Where

ε: molar absorption coefficient

A: specific absorption coefficient

MM: molecular mass

Table 3, summarizes the molar absorption coefficients used for carotenoids quantification.

Table 3. *Molar absorption coefficients of the main carotenoids. EtOH: ethanol; Hx: hexane*

Carotenoids	**ε**	**λ (nm)**	**Solvent**
β-carotene	140400	450	EtOH
β-cryptoxanthin	135700	450	Hx
Lutein	144800	445	EtOH
Zeaxanthin	140900	450	EtOH
Anteraxanthin	137200	446	EtOH
Violaxanthin	153000	440	EtOH

Source: Britton et al. (1995)

Carotenoids quantification was performed by using calibration curves of standards solutions. The concentrations were calculated according to the following formula.

$$C\ (\mu g/\mu l) = [b + (m * A)] * d$$

Where

C: concentration

b: intercept of the calibration curve

m: slope of the calibration curve of the standard X

d: factor considering both analyzed and injection volumes

Liquid chromatography-electrospray ionization ion trap/time of flight mass spectrometry (HPLC-ESI/TOF-MS) analysis

Identification of retinyl esters with long-chain fatty acids was done by HPLC-ESI/TOF-MS. The chromatographic separations were carried out using a Dionex Ultimate 3000RS U-HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The HPLC conditions were the same as described above. An YMC C_{30} column was used (5µm, 250 x 4.6 mm) (YMC, Wilmington, NC, USA). A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer electrospray ion source. Mass spectrometry was carried out on a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with Q-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode using a scan range from *m/z* 50 to 1200. Mass spectra were acquired in MS fullscan. The instrument control was performed using Bruker Daltonics HyStar 3.2. Preliminary identification of the retinyl esters was based on accurate mass, isotopic pattern and fragmentation profile of the molecular ion. Figure 8 and Table 4 contain the high accuracy measurements obtained from the HPLC and ESI mass spectra and the post-processing routine applied with the software.

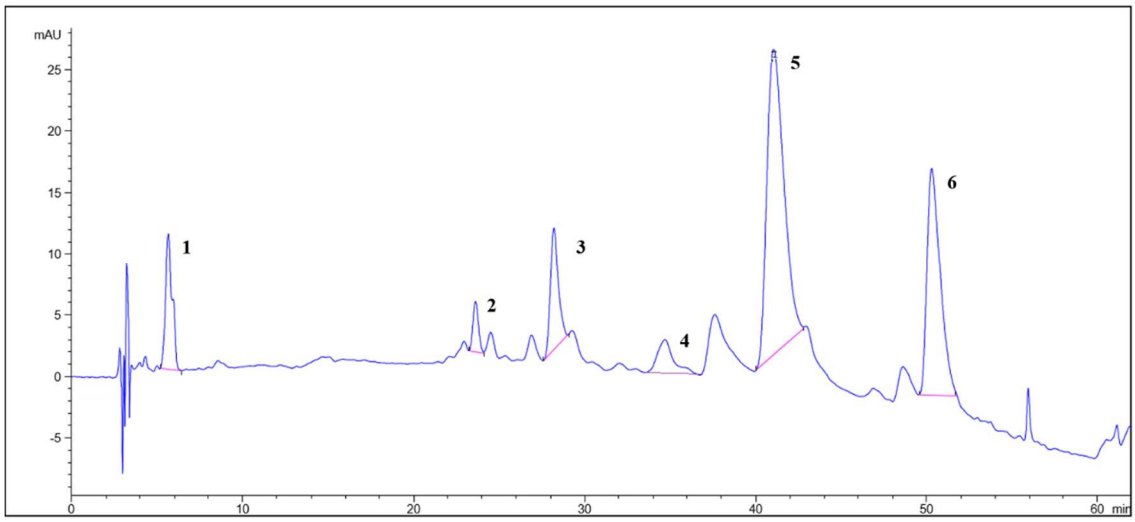


Figure 8. *Typical HPLC chromatogram of the liver fraction*

Table 4. *High accuracy measurements that show the assigned formulas of the retinyl esters identified*

Peak	**R_t (min)**	**Compound identity**	**HPLC-PDA Spectrum λ_{max} (nm)**	**MW. Cal.**	**MW. meas.**	**Error (ppm)**
1	5.5	(all-*t*)-retinol	325	286.4516	n.a.	n.a.
2	22.8	Retinyl linolenate	325	546.4628	547.4727	101.2
3	27.3	Retinyl linoleate	325	549.4868	549.4895	2.7
4	33.8	Retinyl oleate	325	551.5028	551.5057	2.9
5	39.9	Retinyl palmitate	325	525.4868	525.4638	23.0
6	49.7	Retinyl stearate	325	553.5188	553.4951	23.7

R_t means Retention Time. n.a. means not applicable, identified with standard by HPLC-PDA. MW.cal. Means molecular weight calculated for [M + H] . MW.meas. means molecular weight measured for $[M + H]^+$ Error is mean value corresponding to the high accuracy measurements of all isomers on each retinyl ester

Colour measurement in animal adipose tissue

The International Commission on Illumination (C.I.E) recommended in 1976 the international system CIE 1976-(L* a* b*) (CIELab) as the most appropriate for the specification of colour in the food industry because of its uniformity, precision and accuracy. CIELab colour space is a coordinate system defined by three rectangular colorimetric coordinates L*, a*, b* dimensionless parameters (CIE, 2004).

Coordinate L* is defined as lightness and shows values from 0 (black) to 100 (white). Colorimetric coordinates a* and b* form a perpendicular plane to L*. Coordinate a* defines the deviation from the achromatic point, i.e. lightness point, towards red if a* > 0 or green if a* < 0. Likewise, coordinate b* defines the deviation towards yellow if b* > 0 or blue if b* < 0, as it is shown in Figure 9.

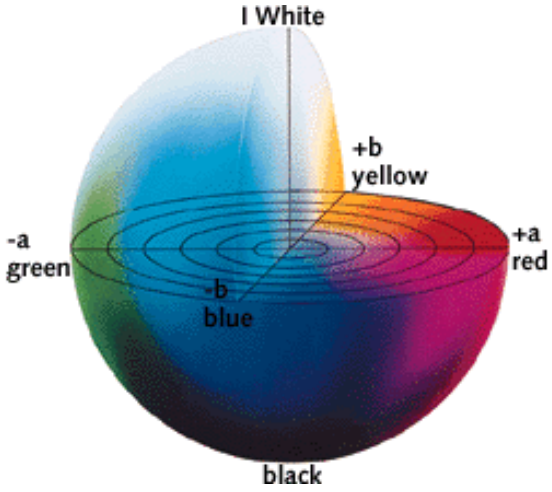


Figure 9. *CIELab colour space*

From these colorimetric coordinates two magnitude are defined, chroma and hue. Chroma, C^*_{ab}, is the attribute of visual perception according to an area which appears to be saturated relative to a certain colour. Hue, h_{ab}, is the qualitative attribute of the colour of an object (blue, yellow, etc) defined by its angular position in a cylindrical space of

colour. It takes values, therefore, between 0º and 360º. These two magnitude are determined mathematically from a* and b* according to the following formulas:

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

$$h = \arctan \frac{b^*}{a^*}$$

A CM-700d spectrocolorimeter (Konica Minolta Holdings, Inc, Osaka, Japan) with D_{65} Illuminant, the 10º Observer and zero and white calibration was used for adipose tissue colour measurements (L*, a*, b*, C^*_{ab} and h_{ab}).

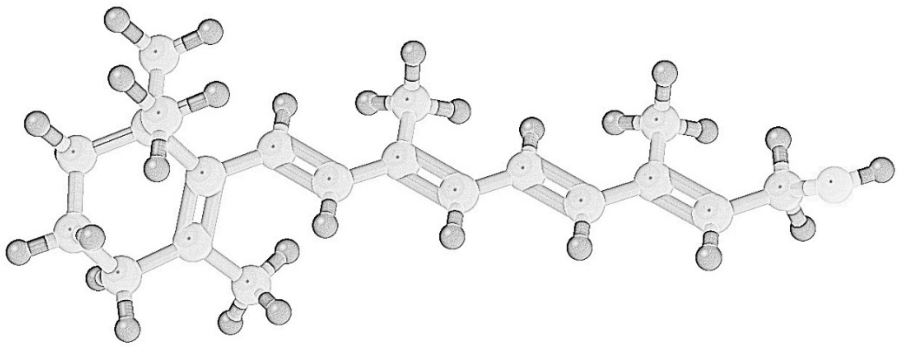
Figure 10. *CM-700d spectrocolorimeter*

Besides, the reflectance spectra on the visible region (approx.. between 360 and 740 nm, considering 10 nm increments) were also acquired and recorded in order to obtain translated reflectance values (TR_i) and the Absolute Value of the Integral (AVI) of the translated spectra. Additionally, in previous studies (Priolo et al., 2002; Ripoll et al., 2008; Zawadzki et al., 2013) the reflectance spectra between 510 and 450 nm, i.e. the region

where carotenoids absorb light, were translated to make the reflectance value at 510 nm equal to zero (TR). The TR_i were calculated from the reflectance values (R_i) as follows: $TR_i = R_i - R_{510}$ with i = 360, 370, 380…740; whereas AVI of the translated spectra were calculated according to the following formula:

$$AVI = [(TR_{450}/2) + TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + TR_{510}/2] \times 10$$

RESULTS AND DISCUSSION



Chapter 1: *Fatty Acid profile and vitamin A in goat milk: influence of the animal's diet*

ANTECEDENTES

Los productos lácteos suponen una fuente vital de proteínas y vitaminas en la nutrición humana. Así, la leche de cabra, en concreto, posee un valor nutricional excepcional. La dieta de los animales tiene un gran impacto en la composición de la leche (Larsen et al., 2012), de forma que una dieta basada es pastos, en comparación con una basada en piensos, resulta en leche con mayores niveles de Ácidos Grasos Poliinsaturados (AGPI) así como de carotenoides y vitaminas liposolubles específicas. Además, la pulpa y la piel de naranja son importantes fuentes de carotenoides (Agócs et al., 2007). En el proceso de producción de zumo de naranja industrial se genera una gran cantidad de subproductos ricos en carotenoides, como la pulpa, la piel o las semillas (Rezzadori et al., 2012). Por esta razón, Rezzadori et al. (2012) propusieron el uso de estos subproductos de la industria del zumo en la alimentación del ganado, ya que podrían conferir un valor añadido a la calidad de los productos animales y, así, suponer una ventaja tanto para la industria del zumo como para el sector de la producción animal.

Por último, en la bibliografía hay información disponible respecto a los niveles de carotenoides en plasma para ovejas, corderos y ganado vacuno pero existen pocos estudios al respecto para el ganado caprino. Por este motivo, sería conveniente realizar estudios para entender los factores que intervienen en los diferentes procesos bioquímicos y fisiológicos que median la absorción, deposición y metabolismo de los carotenoides en las tres especies de rumiantes (Yang et al., 1992).

OBJETIVOS

El principal objetivo de este estudio fue evaluar el efecto de tres dietas diferentes; pasto, dieta enriquecida con pulpa de naranja y concentrado; en:

- Los niveles de carotenoides y retinol en la leche de cabra.
- El perfil de ácidos grasos de la leche de cabra.
- Los niveles de carotenoides y retinol en plasma caprino.

DISEÑO EXPERIMENTAL

Se dividieron cuarenta y cinco cabras de la raza *Florida* en tres grupos según su dieta: el Grupo 1 (G1) eran cabras estabuladas con una dieta basada en alimento concentrado; los animales del Grupo 2 (G2) recibieron una dieta compuesta por alimento concentrado mezclado (TMR, en inglés Total Mixed Ration) y un suplemento de 20% de pulpa de naranja fresca; las cabras del Grupo 3 (G3) fueron alimentadas con pasto. Se recogieron muestras de alimento, plasma y leche de cada grupo.

La extracción de carotenoides y retinol en los alimentos se realizó de acuerdo a la metodología descrita en la bibliografía (Kean et al., 2007; Panfili et al., 2004). Para las extracciones en plasma se siguió la metodología descrita por Lyan et al. (2001). Por último, Por último, las extracciones en leche se hicieron considerando diferentes métodos descritos por otros autores para leche de diferentes especies (Hulshof et al., 2006; Jewell et al., 2004; Nozière et al., 2006b). El contenido en carotenoides y retinol de todas las muestras fue analizado por HPLC. Además, la separación y cuantificación de los ésteres de metilo de los ácidos grasos (AG) se hizo con cromatografía de gases de acuerdo a la metodología descrita por Sukhija & Palmquist (1998).

RESULTADOS

Se encontraron diferencias cuali y cuantitativas en el perfil de carotenoides de las tres dietas estudiadas. También se encontraron diferencias en el perfil de ácidos grasos de las tres dietas.

Respecto a las muestras de los animales, en leche sólo se encontró retinol mientras que en plasma se detectó retinol y luteína. Los niveles más altos ($P<0.001$) de retinol en leche se detectaron para los animales alimentados con pasto y aquéllos que recibieron un suplemento de pulpa de naranja con la dieta. Por lo tanto, se podría afirmar que la leche de cabra con una dieta rica en carotenoides, es decir, aquella basada en pasto o con suplemento de pulpa de naranja, podría ser útil desde un punto de vista nutricional, así como de cara al consumidor, ya que mostró niveles de retinol más altos que la leche de cabra con una dieta basada en alimentos concentrados. Además, el único carotenoide detectado en plasma fue la luteína, la cual fue detectada en los animales alimentados con pasto (G3) y concentrado (G1), mientras que no fue detectada en los animales que recibieron la suplementación con pulpa de naranja. Por último, se observaron diferencias significativas ($P <0.05$) en los niveles de retinol en plasma entre los tres grupos de animales, apareciendo los niveles más altos de retinol circulante en el grupo de animales alimentados con concentrado.

Con respecto al perfil de AG en la leche, los niveles de la fracción n-3 fueron mayores ($P <0,001$) en los animales con una dieta basada en pastos que en los otros dos grupos. Sin embargo, la fracción de CLA no mostró diferencias significativas entre los grupos. Además, los niveles de 9-cis CLA y 11-trans (RA) resultaron más altos ($P <0,001$) en la leche de las cabras alimentadas en pastoreo que en los otros dos grupos. Por otra parte, no se encontraron diferencias significativas en el contenido de AGPI entre G1 (concentrado) y G3 (pasto), pero la cantidad de estos compuestos fue mayor ($P <0,001$)

en estos dos grupos en comparación con el contenido en el G2 (suplementación con pulpa de naranja). Finalmente, la fracción de Ácidos Grasos Saturados (AGS) fue significativamente mayor (*P <0,001*) en la leche de los animales que recibieron suplementación de pulpa de naranja (G2) que en los otros dos grupos (G1 y G3).

Mediante un análisis discriminante se evaluó el efecto de la dieta sobre el nivel de retinol en la leche. El 100% de las cabras con una dieta a base de concentrado (G1) fueron correctamente clasificadas en su grupo según su alimentación, mientras que en los grupos de animales alimentados con pasto (G3) y TMR suplementado con pulpa de naranja (G2) sólo la mitad, aproximadamente, de los animales (53,8% y 46,7%, respectivamente) fueron clasificados correctamente. De acuerdo con estos resultados, se podría señalar el potencial de este suplemento en la dieta delas cabras para lograr en la leche niveles de retinol similares a los de la leche de animales alimentados con pasto, que es considerada tradicionalmente como de "calidad superior". Se realizó un segundo análisis discriminante considerando las fracciones de SFA y n-6 / n-3 en la leche, en el cual el 100% de las cabras fueron clasificados correctamente de acuerdo con su dieta. Confirmando el efecto de la dieta del animal en el perfil de AG de la leche.

CONCLUSIONES

Las principales conclusiones que se extraen de los resultados obtenidos en este estudio son las siguientes:

1. Las cabras alimentadas con una dieta rica en carotenoides (pasto o TMR más un suplemento de pulpa de naranja) producen leche con mayor contenido en retinol que las cabras con una dieta basada en alimentos concentrados.

2. El suplemento de pulpa de naranja en la dieta de las cabras alimentadas con TMR tuvo un efecto beneficioso en el contenido de vitamina A en la leche pero no en el perfil de ácidos grasos.
3. La leche de las cabras alimentadas con pasto presentó un perfil de ácidos grasos "saludable", lo cual es importante desde un punto de vista nutricional.
4. Son necesarios más estudios para establecer la capacidad de las cabras para acumular carotenoides de la dieta.

Fatty Acid profile and vitamin A in goat milk: influence of the animal's diet

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ABSTRACT

The aim of this report was to assess the effect of different diets (concentrate, pasture and diet enriched with orange pulp) on carotenoids and retinol levels in goat milk, as well as on the Fatty Acid (FA) profile. Carotenoids and retinol levels in goat plasma were also studied. Higher retinol levels (*P<0.001*) were found in milk from goats fed an enriched orange pulp diet and grazing goats (1.02 ± 0.43 and 1.18 ± 0.51 µg/mL, respectively) than in milk from goats fed on concentrate (0.23 ± 0.18 µg/mL). Furthermore, n-3 FA fraction was higher (*P<0.001*) in grazing goats than in the other two feeding system . PUFA and MUFA fractions were higher (*P<0.001*) in milk from animals fed on concentrate or pasture than in those fed with orange pulp supplements. Both pasture and orange pulp supplemented diets are interesting from the nutritional point of view, as they would improve vitamin A content in milks conferring an added value to dairy goat products. Additionally, milk from goats fed on pasture showed a better and healthier FA profile but the supplementation of orange pulp did not show any improvement on the FA profile in milk.

INTRODUCTION

Billions of people around the world consume milk and dairy products every day so dairy products suppose a vital source of nutrition (Muchlhoff et al., 2013). Goat milk has an exceptional nutritional quality and it is well tolerated by individuals sensitive and allergic to cow milk and has a benefit effect on health and a high digestibility (Kondyli et al., 2007). Besides, it has been reported (Larsen et al., 2012) that the type and amount of herbage consumed by the animals have an impact on milk composition, so that high levels of grass compared with concentrates based on cereals result in higher milk concentrations

of polyunsaturated fatty acids (PUFA) and conjugated linoleic acid (CLA), as well as carotenoids and fat-soluble vitamins such as retinol or tocopherols. Additionally, it is known (Delgado-Pertiñez et al., 2013) that milk from goats fed on concentrates is rich in Saturated Fatty Acids (SFA) and poor in PUFA. Finally, concentrate feedstuffs, which are usually composed by cereals, tends to present very low levels of carotenoids and fat-soluble vitamins since their oxidation may occur among the manufacture of these concentrate rations (Nozière et al., 2006; Dunne et al., 2009). Moreover, it is known (Agócs et al., 2007) that orange pulp and peel are important sources of carotenoids, including the pro-vitamin A carotenoid β-cryptoxanthin. However, during orange juice production, only around the half of the fresh orange weight is transformed into juice (Rezzadori et al., 2012), producing great amount of carotenoid-rich waste; including pulp, peel or seeds. For this reason, an alternative use for these by-products of the orange juice industry, such as to produce ingredients for animal feed has been proposed (Rezzadori et al., 2012). So that, it could help to add nutritional quality to the animal products and, in this way, present advantages for both orange juice industry and animal production sector.

Finally, several information is available on the carotenoids levels in plasma of sheep, lambs and cattle, but few studies have been conducted to explore the profile of these fat-soluble pigments in goats (Yang et al., 1992). Besides, Yang et al. (1992) suggested that the three ruminant species have different biochemical and physiological pathways for dealing with carotenoid absorption, deposition and metabolism. Thus it would be interesting to compare the deposition of these compounds in goat tissues.

In consequence, the aim of this report was to study the effect of three different carotenoid-containing diets, i.e. pasture, a diet enriched with orange juice by-products and concentrate, on plasma and milk carotenoid and retinol levels in goats. In addition,

the effect of the three types of diets studied on the fatty acid profile of milk was also assessed.

MATERIAL AND METHODS

Animals and diets

Forty-five female goats, of *Florida* breed from three herds in commercial farms, with different feeding system from Southwest of Spain were selected (3 × 15). Animals in group 1 (G1) were fed in confinement *ad libitum* with a diet based on a dry concentrate which was composed by soy (42%), barley (14%), maize (16%), beet pulp (10%), wheat (9%) and rapeseed meal (9%). Those in group 2 (G2) were fed, also indoors, with a total mixed ration (TMR) containing Ray Grass, i.e. *Lolium spp*. (20%), beet pulp (18%), barley (16%), soybean meal (8%), cottonseed (8%), maize (3%) and mineral corrector (7%) and an additional supplement of 20% of orange pulp, in this case the feed was administrate also *ad libitum*. Group 3 (G3) consisted of grazing goats fed on natural pasture. The paddock where the goats were reared was mainly composed of plants from *Poaceae* family, like *Agrostis spp., Vulpia spp., Poa spp., Bromus spp.* and *Lollium spp.* Besides, species from *Fabaceae* family were also present, like *Trifolium spp., Ornithopus spp., Medicago spp*. and *Lotus spp*. These goats were grazing an average of 8 hours per day. Besides, these animals received supplementation along the first month of lactation because of production necessities but not during the rest of the milking period. For this study, animals were selected in the middle of their third or fourth lactation with a diet adapted to their production needs. Milk samples were collected in the same conditions for the three herds, it means in the twelfth week of lactation. The mean milk production levels of the animals, at that moment, were 2.5 ± 0.4 L/day for goats of G1, 2.2 ± 0.2 L/day for G2 and 1.9 ± 0.3 L/day for animals of G3. Goats had an average weight of 74 ± 2 kg.

Sampling

Representative samples of the different diets were taken by triplicate from the farms. In the case of G3, the sampling areas were randomly selected at the prairie where the animals were reared. The pasture was sampled from the ground; to do this three fixed sampling quadrates (0.5 m by 0.5 m) were randomly established. Finally, pasture samples were characterised overall according to the majority herbaceous species present at the gender level. After collecting, all food samples were transported to the laboratory and, then, they were lyophilized and stored at -80 ºC until analysis.

Six millilitres (mL) of blood were taken from the jugular vein of each animal using Li-Heparin as anticoagulant. The blood samples were centrifuged (1500 g, 10 min, 4ºC) and the plasma collected and stored at -80ºC until analysis. What is more, for each group, two aliquots of both morning and afternoon milking were mixed to obtain a representative sample (Delgado-Pertiñez et al., 2009). In total, 30 mL of milk were collected, aseptically into sterile vials, from each goat and immediately stored at -80 ºC until laboratory analysis. Both plasma and milks samples were taken in the twelfth week of lactation.

Carotenoids and retinol extractions

Feedstuffs

The food samples were analysed by triplicate according to the methodology described elsewhere (Panfili et al., 2004; Kean et al., 2007) with some modifications. 3 grams (g) of sample were lyophilized previously to the analysis. The extractions were carried out on the lyophilized extracts and 15 mL of a mixture hexane/ethanol (1:1, v/v) twice. The organic phases obtained were saponified with 15% ethanolic potassium hydroxide (w/v)

overnight at room temperature in the dark and under a nitrogen atmosphere in order to avoid the degradation of carotenoids. Finally the organic phase was washed several times with the same volume of water than organic phase, using a pH indicator to know when all the potassium hydroxide was removed. After that, the organic phase was collected and dried using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The residue was dissolved in 100 µl of ethyl acetate for HPLC analysis.

Milk

The extraction of carotenoids and retinol from milk was carried out considering the methodology described by other authors in milk samples from different species (Jewell et al., 2004; Hulshof et al., 2006; Nozière et al., 2006b). 15 mL of milk were mixed with 7 mL of water, 3.5 mL of ammonia solution 25% (w/v), 15 mL of ethanol and 15 mL of hexane. Samples were then centrifuged (1500 g, 10 min, 4 ºC) and the upper organic phase collected and extracted twice. The organic phases were pooled and 15 mL of sodium hydroxide in ethanol 15% (w/v) were added. The air of the containers was replaced with nitrogen and the saponification reaction was maintained overnight at room temperature in the dark. Finally, the organic phase was washed several times with water, collected and dried using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The extract was stored at -20 ºC under a nitrogen atmosphere until the moment of the analysis. The residue was dissolved in 35 µl of ethyl acetate for HPLC analysis.

Plasma

The methodology described by Lyan et al. (2001) was used with some modifications. 3 mL of plasma diluted with the same volume of distilled water were deproteinized by

adding 3 mL of ethanol. Carotenoids and retinol were extracted twice with 2 mL of dichloromethane and the extracts were evaporated to dryness with a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain) and stored at -20 ºC under a nitrogen atmosphere until the moment of the analysis. The extracts were dissolved in 35 µl of ethyl acetate and injected on the HPLC system.

HPLC conditions

The analyses were performed on an Agilent 1100 system (Agilent, Palo Alto, CA, United States) fitted with a photodiode array detector, a quaternary pump, a column temperature control module set at 20 °C and housing an YMC C_{30} column (5 lm, 250 x 4.6 mm) (YMC, Wilmington, NC, USA), and an auto sampler set to draw 20 µl aliquots from the concentrated extracts. The mobile phase consisted in methanol (MeOH), methyl-ter-butyl ether (MTBE) and water according to the linear gradient: 0 min: 90% MeOH + 5% MTBE + 5% water; 12 min: 95% MeOH + 5% MTBE ; 25 min: 89% MeOH + 11% MTBE; 40 min: 75% MeOH + 25% MTBE; 50 min: 40% MeOH + 60% MTBE; 56 min: 15% MeOH + 85% MTBE; 62 min: 90% MeOH + 5% MTBE + 5% water. The mobile phase was pumped at 1 mL/min and the chromatograms were monitored at 450 nm for carotenoids and at 325 nm for retinol The carotenoids detected were identified by comparison of their spectroscopic and chromatographic characteristics with those of standards obtained from natural sources by recommended procedures as described elsewhere (Meléndez-Martínez et al., 2009). The retinol peak was identified by using a standard from Sigma Chemical (Madrid, Spain). The quantification was performed by using calibration curves of standard solutions.

Fatty Acids analysis in feedstuffs and milk

Separation and quantification of Fatty Acid (FA) methyl esters were carried out using a gas chromatograph Agilent 6890N Network GS System (Agilent, Santa Clara, CA), equipped with a flame-ionization detector and fitted with an HP-88 capillary column (100 m, 0.25 mm i.d., 0.2 µm film thickness). Nonanoic acid methyl ester (C9:0 ME) at 4 mg/mL was used as an internal standard. Extraction and direct methylation were performed in a single-step procedure based on the method published previously (Sukhija et al., 1998). Individual FA were identified by comparing their retention times with those of an authenticated standard FA mix Supelco 37 (Sigma Chemical Co. Ltd., Poole, UK). Identification of the conjugated linoleic acid (CLA) isomers *cis*-9, *trans*-11, was achieved by comparing retention times with those of another authenticated standards (Sigma Chemical Co. Ltd.). FA content was expressed as the percentage of total methyl esters identified and grouped as follows: SFA, MUFA, and PUFA.

Statistical analysis

The SPSS 15.0 for Windows (SPSS Inc., 2006) software was used. Analysis of variance (ANOVA) test was applied to assess the existence of significant differences among the three groups of animals. The significant differences between group means were determined by a Tukey-b post hoc test, with a significance level of $P < 0.05$. Correlations analyses were performed between retinol and FA in milk and lutein and retinol in plasma using the GLM procedure of SPSS. Finally, two discriminant analysis /DA) were carried our using a stepwise model considering the diet of the animals as independent variable. The discriminant classification method was leave-one-out-cross-validation. The first DA was based on retinol milk levels and the second one on the FA profile of milk. The significance level for a variable to be included in the model was 0.05.

RESULTS AND DISCUSSION

Carotenoid and fatty acid profile in feedstuffs

Table 5 summarizes the information relative to the main carotenoids present in the feedstuffs. Both lutein and β-carotene were detected in different proportions in all the food samples but not in the orange pulp, finding the lowest amounts in the concentrate and the highest in the pasture. These results are in accordance with previous findings by other authors (Cardinault et al., 2008; Prache, 2009), who reported lutein and β-carotene as the main carotenoids in fresh pasture. Besides, it has been previously described (Nozière et al., 2006a; Röhrle et al., 2011) the presence of lutein, zeaxanthin and β-carotene for cereal-based concentrate diets, which is in accordance with the present study (Table 1) since concentrate diets were, also, based on cereals. In addition to these compounds, antheraxanthin and β-cryptoxanthin were also found in the diets of G1 and G2. The amount of carotenoids was always, significantly higher ($P<0.001$) in the samples corresponding to G2, probably due to the presence of Ray Grass in these feedstuff samples Considering these results we can state that there were both qualitative and quantitative differences in the carotenoid profile of the three diets received by the animals in the present study.

Table 5. *Differences on carotenoids concentration (µg/g DM ± SD) in goat's feed: concentrate (G1), TMR (Total Mixed Ration) + orange pulp (G2) and pasture (G3).*

	Concentrate (G1)	TMR (G2)	Orange pulp (G2)	Pasture (G3)	Sig.
Lutein	0.94^{a} ± 0.10	1.86^{ab}± 1.43	n.d.	4.06^{b}± 0.60	*
Zeaxanthin	0.73 ± 0.42	0.63 ± 0.02	1.01 ± 0.07	n.d.	n.s.
Anteraxanthin	0.65 ± 0.29	n.d.	7.18 ± 0.70	n.d.	**
β-cryptoxanthin	0.38^{a}± 0.01	0.82^{a}± 0.07	22.30^{b}± 2.57	n.d.	***
β-carotene	0.34^{a}± 0.07	7.61^{a}± 6.11	n.d.	41.31^{b}± 7.21	**

Sig.: significant differences. *$P<0.05$, **$P<0.01$, ***$P<0.001$, n.s.: not significant. Significant differences ($P<0.05$) within a row are indicated by superscripts (a, b). n.d.: not detected

In Table 6 it can be observed the FA profile of the feedstuffs analysed in this study. According to Woods & Fearon (2009) the main FA present in grass-forage supplied to ruminants are palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1 n-9 *cis*), linoleic acid (C 18:2 n-6 *cis*) and α-linolenic acid (C 18:3 n-3), which is in accordance with the FA profile of the pasture samples analysed in this study (Table 6). In addition, Chilliard et al. (2001) stated that silage and hay making process can influence FA concentration reducing both FA levels in general and α-linolenic acid amounts in grass processing, which could explain the lower percentage of α-linolenic in TMR than in pasture . Finally, Brat et al. (2003) reported palmitic, stearic, oleic, linoleic and α-linolenic as the main FA in orange pulp, our data partially agree with these authors since oleic acid was not detected in orange pulp (Table 6).

Table 6. *Fatty acid profile (% of total fatty acids) of the feedstuffs of three groups of goats: G1 (concentrate), G2 (Total Mixed Ration + Orange pulp), G3 (Pasture)*

Fatty acid[a]	**Concentrate (G1)**	**TMR (G2)**	**Orange pulp (G2)**	**Pasture (G3)**
C8:0	0.07	0.20	0.00	0.10
C10:0	0.07	1.28	0.00	0.12
C12:0	0.08	0.73	0.00	0.20
C14:0	1.25	2.41	0.00	0.72
C15:0	0.11	0.19	0.00	0.18
C16:0	20.94	25.23	39.14	29.92
C16:1 n-9	1.61	0.26	0.58	1.50
C17:0	0.34	0.23	0.00	0.22
C17:1	0.22	0.12	0.00	0.07
C18:0	8.21	13.64	8.47	20.18
C18:1 n-9 cis	30.09	19.51	0.00	1.14
C18:2 n-6 trans	0.07	0.08	0.00	0.07
C18:2 n-6 cis	31.71	32.70	28.43	7.32
C18:3 n-6	0.02	0.05	0.16	0.26
C20:0	0.26	0.41	0.00	0.00
C18:3 n-3	2.23	1.82	10.32	34.67
C20:1 n-9	0.68	0.28	0.14	0.14
C21:0	0.03	0.08	0.07	0.06
C20:2	0.25	0.09	0.00	0.11
C22:0	0.05	0.29	0.00	2.25

C20:4 n-6 (ARA)	0.20	0.13	0.00	0.12
C20:3 n-3	1.34	0.07	0.00	0.04
C20:5 n-3 (EPA)	0.06	0.10	0.00	0.13
C22:5 n-3 (DPA)	0.07	0.05	0.00	0.32
C22:6 n-3 (DHA)	0.04	0.05	0.00	0.18
SFA	31.41	44.68	47.68	53.94
MUFA	32.61	20.18	13.41	2.86
PUFA	35.98	35.14	38.92	43.21
SFA/PUFA	0.87	1.27	1.23	1.25
SFA/UFA	0.46	0.81	0.91	1.17
n-3	3.75	2.09	10.48	35.33
n-6	31.99	32.96	28.59	7.77
n-6/n-3	8.54	15.80	2.73	0.22

[a]ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid (FA); MUFA, monounsaturated FA; PUFA, polyunsaturated FA; UFA, unsaturated FA; n-3, all FA with last double bond at 3rd carbon from methyl end; n-6, all FA with last double bond at 6th carbon from methyl end.

Carotenoids and retinol levels in milk and plasma

In milk, only retinol was found while in plasma both retinol and lutein were detected (Table 7). Despite the high level of β-carotene present in the feedstuff, the absence of β-carotene in milk can be explained by the high enzymatic conversion of β-carotene to retinal (precursor compound of retinol) by the 15,15'-dioxygenase in the goat intestine.

In fact, β-carotene was not either found in plasma, which could explain its absence in the milk, in agreement with previous studies (Lucas et al., 2008b). The highest retinol levels in milk were detected in grazing animals and in those fed with TMR plus orange pulp, being its concentration fivefold ($P<0.001$) higher than in milk from animals fed on concentrate. Previous studies (Fedele et al., 2004: Morand-Fehr et al., 2007) reported that retinol content in milk from grazing goat was higher (often significantly) than in milk from animals on an intensive regime. However, to our knowledge, supplementing the diet with orange pulp had not been evaluated previously. It has also been reported elsewhere (Lucas et al., 2008a; Yang et al., 2010) that the amount of retinol in milk is directly dependent on the level of provitamin A (in particular β-carotene) in the diet, as it can be expected beforehand. This fact is also observed in this study as the highest amount of retinol in milk were detected in the goats from the group receiving pasture and those receiving TMR plus orange pulp. Therefore, it could be claimed that milk from goats with a diet rich in carotenoids, i.e. pasture or supplemented with orange pulp, could be useful from a nutritional and consumer's point of view, since it showed higher retinol levels than milk from goats with a diet based on concentrate.

Table 7. *Lutein and retinol in goat milk and plasma (µg/mL ± SD) in the three groups of animals. G1: goats fed on concentrate, G2: goats fed on TMR (Total Mixed Ration)+ orange pulp and G3: goats fed on pasture.*

	Variable	G1	G2	G3	Sig.
Plasma	Lutein	0.01 ± 0.00	n.d.	0.04 ± 0.03	*
	Retinol	1.18^{b}± 0.84	0.64^{ab}± 0.37	0.22^{a}± 0.09	*
Milk	Lutein	n.d.	n.d.	n.d.	-
	Retinol	0.23^{a}± 0.18	1.02^{b}± 0.43	1.18^{b}± 0.57	***

Sig.: significant differences. *$P<0.05$, ***$P<0.001$. Significant differences ($P<0.05$) within a row are indicated by superscripts (a, b). n.d.: not detected.

On the other hand, the only carotenoid detected in plasma was lutein (Table 3). This observation is in accordance with previous studies in sheep and goats (Yang et al., 1992; Nozière et al., 2006a). Nevertheless, lutein was not either detected in plasma from goats fed on TMR + orange pulp (G2), in spite of lutein was present in their diet (Table 5). In principle, it could be thought that the absence of lutein in these samples could be due to the absence of this compound in the orange pulp but it has to be considered that the percentage of pulp in the diet of these animals was only 20%. In this regard, more studies are needed to determine the cause of the lack of lutein in the plasma of these animals. Finally, significant differences ($P<0.05$) in plasma retinol levels among the three groups of animals were observed, showing the highest circulating retinol levels those goats fed on concentrate (Table 7).

According to the results discussed above, it can be stated that grazing animals showed the lowest amount of retinol in plasma but the highest in milk. It has been hypothesized by previous authors (Schweigert et al., 1990; Calderón et al., 2007) that, in cattle the conversion of β-carotene into retinol by the 15,15'-dioxygenase is also produced in the

mammary gland, causing a rise in the retinol concentration in milk and, as a consequence, a decrease in plasma. These authors observed that the parenteral administration of β-carotene was followed by an increase of retinol concentration in milk but not in plasma. Besides, it can be noticed that, according to our results, retinol levels in milk from animals fed on pasture and those fed on TMR plus orange pulp are not different (Table 7), indicating that supplementing goat diet with orange pulp makes no difference in retinol or other carotenoid levels. Therefore, it could be pointed out the potential of supplementing the diet with orange pulp in order to achieve retinol levels similar to those in the milk from animals feed on grazing, which is traditionally considered as "higher quality".

Fatty acid profile in milk

15-20% of the total fat intake of humans, 25% of which is saturated fat, is derived from dairy products (Elgersma et al., 2006) and the FA composition depends to a large extent on the diet composition fed to the animals (Silanikove et al., 2010). For this reason, current research is focusing on n-3 FA and Conjugated Linoleic Acid (CLA) which benefits human health (Mancilla-Leytón et al., 2013). In this sense n-3 fraction level in milk was higher ($P<0.001$) in animals with a diet based on pasture (G3) than in the other two groups (Table 4) which is in accordance with previous studies (D'Urso et al., 2008; Mancilla-Leytón et al., 2013). However, the CLA fraction did not show significant differences (Table 8) between groups, i.e. the feeding system did not influence that fraction in milk, as reported previously by Mancilla-Leytón et al. (2013). Besides, CLA *cis*-9, *trans*-11 (RA) has been established as the most predominant CLA compound in milk (Lock et al., 2004), which is in accordance with the data obtained in this study (Table 8), being its amount higher ($P<0.001$) in milk from goats fed on pasture than in milk from

the other two feeding groups. CLA may be beneficial to human health especially for its anticarcinogenic activity and food products coming from ruminants are considered as the main source of CLA in human diets (Lock et al., 2004).

In addition, Mancilla-Leytón et al. (2013) also reported in their trial a similar content of PUFA in milk between grazing and indoor-fed with concentrate goats, which also agree with our data since no significant differences were found (Table 8) between G1 and G3 in this sense Besides, under our trial conditions, the n-6/n-3 FA ratio was in accordance with current human health recommendations, 2.0-2.5 (MacRae et al., 2005), (Table 4), for all feeding systems studied. Considering all these data we agree with Silanikove et al. (2010) who affirmed that milk from goats fed on pasture may present an overlooked "treasure trove" with respect to its health promoting lipid profile and its beneficial consequences to human health. Furthermore, it has been reported (Silanikove et al., 2010) that goat fat milk contains a high proportion of medium-chain FA, i.e. caproic (C6: 0), caprylic (C8: 0) and capric (C10: 0), which are partly responsible for the characteristic "goaty" odour of goat milk. These compounds were detected in all milk studied in this report (Table 4) without significant differences in their amount among the three groups, except for capric acid which was present in higher ($P<0.001$) amount in milk from goats fed on TMR + orange pulp supplementation (G2) than in the other two groups. Finally, in Table 8 it can be observed that SFA fraction, as well as SFA/PUFA and SFA/UFA ration in milk from G2 were significantly higher ($P<0.001$) than in the other two groups. In addition, PUFA, n-3 and n-6 fractions were significantly lower ($P<0.001$) in goat milk from G2 than in the other two groups. Therefore, we can conclude that a diet based on TMR with a supplementation of orange pulp for goats did not improve the FA profile in milk.

Table 8. *Fatty acid profile (% of total fatty acids) of the milk in Florida goats of three groups of animals: G1 (goats fed on concentrate), G2 (goats fed on Total Mixed Ration + Orange pulp), G3 (goats fed on pasture).*

Fatty acid[a]	**G1**	**G2**	**G3**	**SEM**[b]	**Sig.**
C4:0	1.96	1.95	1.78	0.04	**n.s.**
C6:0	2.07	2.03	1.90	0.04	**n.s.**
C8:0	2.23	2.13	2.01	0.04	**n.s.**
C10:0	8.85a	13.36b	8.68a	0.50	***
C11:0	0.06a	0.11b	0.10b	0.01	***
C12:0	3.39a	5.86b	3.75a	0.26	***
C13:0	0.05a	0.08b	0.09b	0.01	***
C14:0	7.45a	9.95b	7.39a	0.27	***
C14:1	0.16a	0.20b	0.53c	0.04	***
C15:0	0.46a	0.75b	0.98c	0.05	***
C15:1	0.02a	0.07b	0.03a	0.01	***
C16:0	25.71b	32.54c	24.48a	0.81	***
C16:1 n-9	1.27c	0.67a	1.00b	0.06	***
C17:0	0.58a	0.57a	0.68b	0.01	***
C17:1	0.31b	0.25a	0.25a	0.01	***
C18:0	13.84b	11.09a	16.28c	0.49	***
C18:1 n-9 trans	1.89b	0.41a	0.49a	0.16	***
C18:1 trans-11 (VA)	1.75b	0.88a	0.84a	0.10	***
C18:1 n-9 cis	22.72b	13.74a	23.96c	1.02	***
C18:2 n-6 trans	0.26a	0.29a	0.59b	0.03	***
C18:2 n-6 cis	2.91c	1.33a	1.92b	0.15	***
C18:3 n-6	0.06	0.07	0.05	0.01	**n.s.**
C20:0	0.42c	0.23a	0.36b	0.02	***
C18:3 n-3	0.21a	0.22a	0.40b	0.02	***
CLA cis-9. trans-11 (RA)	0.29b	0.22a	0.49c	0.03	***
C20:1 n-9	0.23c	0.08a	0.10b	0.02	***
CLA trans-10. cis-12	0.11b	0.08a	0.12b	0.01	***
C21:0	0.05a	0.06a	0.10b	0.01	***
C20:2	0.06a	0.05a	0.08b	0.01	***

Table 8 continuation. *Fatty acid profile (% of total fatty acids) of the milk in Florida goats of three groups of animals: G1 (goats fed on concentrate), G2 (goats fed on Total Mixed Ration + Orange pulp), G3 (goats fed on pasture).*

Fatty acid[a]	**G1**	**G2**	**G3**	**SEM[b]**	**Sig.**
C22:0	0.06b	0.10c	0.04a	0.01	***
C20:3 n-6	0.03b	0.03b	0.02a	0.01	**
C22:1 n-9	0.08b	0.03a	0.02a	0.01	***
C20:4 n-6 (ARA)	0.12b	0.15b	0.07a	0.01	***
C20:3 n-3	0.04b	0.02a	0.02a	0.01	***
C23:0	0.01ab	0.02b	0.01a	0.01	*
C20:5 n-3 (EPA)	0.10	0.10	0.11	0.01	**n.s.**
C22:2	0.08b	0.07b	0.04a	0.01	***
C24:0	0.02a	0.04b	0.03ab	0.01	**
C24:1	0.01a	0.03b	0.02a	0.01	**
C22:5 n-3 (DPA)	0.08a	0.11b	0.10b	0.01	***
C22:6 n-3 (DHA)	0.03a	0.06b	0.07b	0.01	***
SFA	67.21a	80.86c	68.67b	1.37	***
MUFA	28.43c	16.35a	27.24b	1.22	***
PUFA	4.36b	2.79a	4.09b	0.16	***
SFA/PUFA	15.47a	29.08b	16.84a	1.39	***
SFA/UFA	2.05a	4.23c	2.19b	0.22	***
CLA	0.52	0.54	0.57	0.01	**n.s.**
n-3	0.45a	0.51b	0.70c	0.02	***
n-6	3.39c	1.86a	2.66b	0.15	***
n-6/n-3	7.54b	3.67a	3.82a	0.41	***

Mean with different alphabets (a, b and c) among columns differ significantly.

[a] VA, vaccenic acid; RA, rumenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids (FA); MUFA, monounsaturated FA; PUFA, polyunsaturated FA; UFA, unsaturated FA; CLA, total conjugated linoleic acid; n-3, all FA with last double bond at 3rd carbon from the methyl end; n-6, all FA with last double bond at 6th carbon from the methyl end.

[b]Standard Error of Mean.

Moreover, Table 9 summarizes the results obtained for the correlations coefficients of FA fractions and retinol in milk and plasma, and lutein in plasma. It could be observed that the content of retinol in plasma did not show significant correlations with any of the FA fractions analyzed in milk. Whereas, the n-6/n-3 fraction in milk showed (Table 9) significant and negative correlations with both content of lutein in plasma (r = -0.767, $P<0.05$) and retinol levels in milk (r = -0.692, $P<0.01$). In this sense, previous authors (Rafalowski et al., 2014) reported a high positive correlation between the sum of polyenoic and monoeonic FA and the content of vitamin A, among other antioxidants. This affirmation is in disagreement with our data (Table 9). This is probably due to the fact that milk from animals fed on concentrate (G1) showed (Table 8) higher ($P<0.001$) n-6/n-3 fraction and lower amount of lutein in plasma ($P<0.05$) and retinol in milk ($P<0.001$) than the milk from animals with a diet based on TMR + orange pulp supplementation (G2) and goats fed on pasture (G3). Therefore, it could be concluded, that under our trial conditions, there is an effect of the diet on the FA profile in goat milk according to previous studies (Delgado-Pertiñez et al., 2013; Rafalowski et al., 2014), as well as, orange pulp supplementation did not improve the FA profile in goat milk as it was explained above.

Table 9. *Correlation coefficients between retinol and FA in milk and lutein and retinol in plasma.*

	Lutein (plasma)	Retinol (plasma)	Retinol (milk)
Retinol (plasma)	-0.561		
Retinol (milk)	0.535*	-0.403*	
SFA	0.599	-0.263	0.323
MUFA	-0.505	0.262	-0.314
PUFA	-0.567	0.257	-0.381
n-6/n-3	-0.767*	0.194	-0.692**

*Correlation coefficients significant at a level of *P<0.05*

**Correlation coefficients significant at a level of *P<0.01*

Effect of the diet on the retinol levels and FA profile in goat milk

Finally, in order to assess the effect of the diet on the retinol levels and FA profile in milk two discriminant analyses were carried out. The first one considered retinol levels in milk as variable (Table 10). As a result up to 65.9% of the animals were correctly classified into their group according to their diet. More specifically, 100 % of goats with a diet based on concentrate (G1) were accurately classified, whereas in the groups of pasture (G3) and TMR supplemented with orange pulp (G2) just half of the animals (53.8% and 46.7 %, respectively) were correctly classified. According to these results, it could be stated that supplementing goat diet with orange pulp makes no difference in retinol milk levels. Thus, it could be pointed out the potential of this supplementation in the diet to achieve retinol milk levels similar to those in milk from grazing animals, which are traditionally considered as “higher quality” (Prache et al., 2009). Moreover, in those arid areas where pasture are scarce, supplementing TMR with orange pulp could suppose

a nutritional advantage, since vitamin A levels in goat milk were increased .A second discriminant analysis was performed considering the FA profile in milk. SFA and n-6/n-3 fractions were considered in the analysis and the 100% of the goats were accurately classified according to their diet Figure 11). Thus, it could be affirmed, once again, that the FA profile of milk is influenced by the diet of the animals. In addition and according to data in Table 8 the SFA fraction was highly influenced by the diet, as the highest ($P<0.001$) SFA fraction appeared in goats from G2. Therefore, supplementing with orange pulp did not seem useful to enhance the healthy FA profile in goat milk.

Table 10. *Classification matrix after discriminant analysis based on retinol content in milk: assigned (columns) against real data (rows).*

	Predicted group (%)		
Group	*1*	*2*	*3*
1	**100.0**	0.0	0.0
2	20.0	**46.7**	33.3
3	23.1	23.1	**53.8**

Group 1: goats with a diet based on concentrate. Group 2: goats fed on TMR (Total Mixed Ration) and orange pulp supplementation. Group 3: pasture goats.

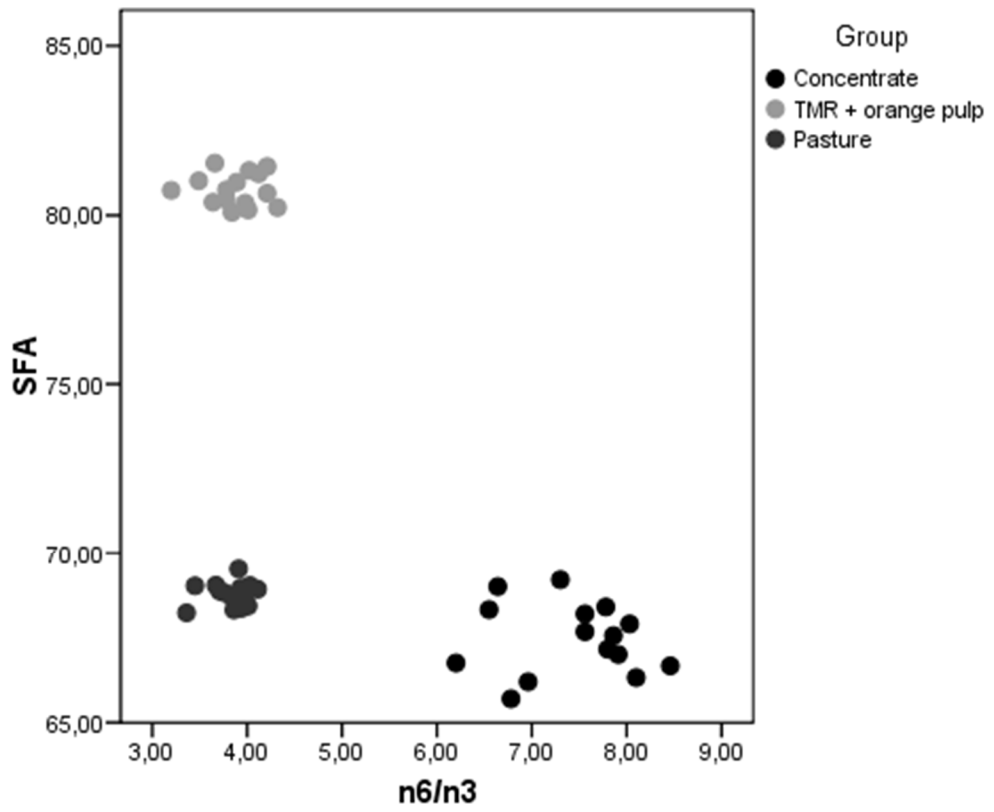

Figure 11. *Classification of goats according to their diet (concentrate, TMR + orange pulp supplementation and pasture) considering SFA and n-6/n-3 fraction in milk*

CONCLUSIONS

Retinol levels in milk from goats fed on a diet rich in carotenoids, pasture or supplemented with orange pulp, are higher than in milk from animals fed on a diet based on concentrate. Additionally it could be claimed that supplementing goat diet with orange pulp in arid areas where pasture are scarce, may increase vitamin A levels in milk. In addition, milk from goats fed on pasture showed a healthier FA profile, with PUFA ratios over 4.09. However, orange pulp supplementation to TMR did not show any improvement on the FA profile in milk.

Chapter 2 *Effect of pasture and concentrate diets on concentrations of carotenoids, vitamin A and vitamin E in plasma and adipose tissue of lambs*

ANTECEDENTES

Actualmente, los consumidores están cada vez más interesados tanto en la "imagen verde" como de las propiedades saludables de los productos procedentes de animales alimentados con pasto (Sheath et al., 2001; Prache et al., 2003b; Nozière et al., 2006ª; Dian et al., 2007a). Por este motivo se están realizando trabajos de investigación sobre la autenticación de la dieta en los productos de animales herbívoros, de modo que se han utilizado ciertos compuestos de la dieta, como carotenoides, para autenticar el sistema de producción (extensivo *vs.* intensivo) de productos de origen animal (Sheath et al., 2001; Prache et al., 2003a; Dian et al., 2007b; Serrano et al., 2007).

Además de los sistemas de producción extensivo e intensivo, el sistema más utilizado en la cría de pequeños rumiantes en los países mediterráneos involucran cabritos y corderos lechales (Bernués et al, 2012; Alcalde et al, 2013). Por ello, podría ser interesante estudiar la existencia o no de correlaciones entre los niveles de carotenoides y otros micronutrientes en el plasma y la leche de la oveja y el plasma del cordero lechal como posible herramienta para autenticar la dieta basada exclusivamente en leche de estos animales.

Además de la presencia de carotenoides en los tejidos animales, Prache y Theriez (1999) propusieron un análisis matemático del porcentaje de reflectancia del tejido adiposo como medida indirecta para discriminar los sistemas de producción, extensivos e intensivos, en corderos. Sin embargo, la concentración de carotenoides en los tejidos animales puede variar ampliamente según su presencia en la dieta (Dian et al., 2007b) y

su persistencia es baja (Prache et al., 2003b). Por lo tanto, sería interesante investigar nuevos compuestos y/o metodologías para diferenciar, individualmente o en combinación con los ya descritos, los sistemas de alimentación en herbívoros con el fin de aumentar la precisión de dicha discriminación.

OBJETIVOS

El objetivo principal de este estudio fue buscar nuevas herramientas de trazabilidad en la alimentación de corderos con el fin de mejorar la precisión en la discriminación de los sistemas de producción extensivo e intensivo en el ganado ovino. Como objetivos específicos se propusieron los siguientes:

- Evaluar del efecto de diferentes dietas en los niveles de carotenoides y vitaminas liposolubles (retinol y α-tocoferol) en diferentes tejidos de ganado ovino para estudiar los posibles vínculos con las condiciones del sistema de producción.
- Evaluar el efecto de diferentes dietas sobre los niveles de carotenoides y vitaminas liposolubles (retinol y α-tocoferol) en diferentes tejidos de ovejas para estudiar posibles vínculos con las condiciones de los sistemas de producción ganadera.
- Evaluar la utilidad de los niveles de carotenoides, retinol y α-tocoferol en combinación con parámetros de medida del color en el tejido adiposo para diferenciar corderos alimentados con pasto de aquellos alimentados con concentrados.
- Estudiar la existencia de correlaciones entre los niveles de retinol, α-tocoferol y carotenoides de la oveja y el cordero lechal con el fin de autenticar la dieta exclusivamente láctea de los corderos.

DISEÑO EXPERIMENTAL

Para este estudio se utilizaron tres grupos de ovejas de la raza *Merino*: 15 corderos alimentados con pasto (G1), 15 corderos con una dieta basada en alimentos concentrados (G2) y 15 corderos lechales (G3) cuyas madres fueron cebadas en un sistema extensivo. Se recogieron muestras representativas de las dietas, incluyendo leche de las madres de los corderos lechales. Además se recogió plasma de todos los animales y grasa perirenal de los corderos de los grupos 1 y 2.

Se midió el color (C^*_{ab}, L*, a*, b* y h_{ab}) de la grasa perirenal de acuerdo al sistema CIELab. Además, se obtuvo el espectro de reflectancia de la región visible y se calcularon los valores trasladados del espectro (TR) y el Valor Absoluto de la Integral (AVI, en inglés) siguiendo la metodología desarrollada por otros autores (Prache & Theriez, 1999; Priolo et al., 2002; Ripoll et al., 2008; Zawadzki et al., 2013). Las extracciones de carotenoides, retinol y α-tocoferol se realizaron siguiendo la metodología descrita previamente en la bibliografía (Kean et al., 2007; Panfili et al., 2004). Para las extracciones en las muestras de leche se tuvo en cuenta la metodología descrita por otros autores en leche de distintas especies (Hulshof et al., 2006; Jewell et al., 2004; Nozière et al., 2006b). En el caso de las muestras de plasma se siguió la metodología descrita por Lyan et al. (2001). Finalmente, las extracciones en las muestras de grasa perirenal se realizaron siguiendo métodos descritos previamente (Dunne et al., 2006; Nozière et al., 2006b). El contenido en carotenoides, retinol y α-tocopherol fue determinado por HPLC en todas las muestras.

RESULTADOS

Se encontraron diferencias significativas tanto en el perfil de carotenoides como en los niveles de α-tocoferol entre pastos y concentradas. Sin embargo, no se detectó retinol en ninguna de las dietas estudiadas. Por lo tanto, se podría esperar que los niveles de estos compuestos en los tejidos animales fueran diferentes entre animales alimentados con pasto y aquellos con una dieta basada en alimento concentrado. Por otro lado, en la leche de las madres del G3 no se encontraron carotenoides pero sí retinol y α-tocoferol. Por ello cabía esperar que ambas vitaminas aparecieran también en el plasma de los corderos lechales y, de esta manera, ayudar a diferenciar su plasma del de los otros corderos (G1 y G2), así como ser útiles para confirmar la dieta exclusiva de leche de estos animales.

En el plasma de los corderos alimentados con pasto (G1) se detectaron tanto luteína como β-caroteno, mientras que el resto de los corderos (G2 y G3) no mostraron carotenoides en su plasma. Sin embargo, en todos los plasmas se detectaron las dos vitaminas liposolubles (retinol y α-tocoferol). Por ello, se evaluó, mediante un análisis discriminante, la utilidad de dichas vitaminas para diferenciar los animales según su dieta, el 61.9% de los animales fueron correctamente clasificados según su dieta. Por último, no se encontraron correlaciones significativas en los niveles de retinol y α-tocoferol entre el plasma de los corderos lechales y la leche de sus madres.

Por otro lado, en la grasa perirenal de los corderos alimentados con pastos (G1) y concentrado (G2) se detectó tanto retinol como α-tocoferol, siendo sus niveles siempre mayores ($P < 0,01$ para retinol y $P < 0,05$ para α-tocoferol) para los animales alimentados en un sistema extensivo (G1) que los criados en un sistema intensivo (G2), lo que implicaría que los productos de ovejas alimentadas con pasto tienen mayores niveles de retinol y α-tocoferol, mejorando su valor para la nutrición humana. Además, se hizo un segundo análisis discriminante con el fin de evaluar la utilidad de los parámetros medidos

en la grasa perirrenal para discriminar los dos principales sistemas de alimentación en los corderos, es decir extensivo basado en pastos e intensivo a base de concentrado. En el modelo entraron los niveles de retinol y α-tocoferol y AVI. Este modelo permitió clasificar correctamente el 100% de los animales de acuerdo a su dieta (pasto *vs*. concentrado). Por lo tanto, habría que destacar que la combinación de estos tres parámetros mejora, bajo nuestras condiciones de ensayo, la fiabilidad de la discriminación entre los sistemas de alimentación para corderos.

CONCLUSIONS

De acuerdo con los resultados obtenidos en este estudio, las principales conclusiones son:

1. Los niveles de luteína y β-caroteno en plasma aparecieron como una herramienta útil para diferenciar los corderos alimentados exclusivamente con pasto de aquellos alimentados con otras dietas.
2. Se necesitan más estudios para establecer la existencia o no de las correlaciones en los niveles de retinol y α-tocoferol entre la leche de oveja y el plasma de los corderos lechales.
3. Un sistema de cría extensivo con una dieta basada en pastos mejoraría el valor de los productos ovinos desde el punto de vista de la nutrición humana, ya que esta dieta confiere mayores niveles de retinol y α-tocoferol a los tejidos de ovejas.
4. Considerando los valores de AVI y los niveles de retinol y α-tocoferol en grasa perirrenal, el 100% de los corderos fueron clasificados correctamente de acuerdo a su sistema de alimentación, es decir, pastos *vs*. concentrado. Por lo tanto, se propone el uso combinado de estos tres parámetros como una herramienta útil para estudios de trazabilidad en la alimentación de corderos.

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Original Research Article

Effect of pasture and concentrate diets on concentrations of carotenoids, vitamin A and vitamin E in plasma and adipose tissue of lambs

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ABSTRACT

The effect of different diets on the content of carotenoids, fat-soluble vitamins (retinol and tocopherol) and color measurements in sheep tissues was assessed, to study the possible links with the livestock production system for both nutrition and traceability. Three groups of animals were considered: 15 lambs fed on pasture (G1), 15 lambs fed on concentrate (G2) and 15 suckling lambs (G3) whose mothers were grass-fed. Lutein and β-carotene appeared only in plasma from grazing lambs, so they appear useful to differentiate lambs fed on pasture. Furthermore, retinol and α-tocopherol content in fat were significantly higher ($p < 0.01$) in the animals fed on an extensive system (1.48 ± 0.08 and 42.4 ± 1.76 μg/g fat, respectively) than those fed on an intensive one (1.03 ± 0.08 and 34.8 ± 0.23 μg/g fat, respectively). When fat-soluble vitamin (retinol and tocopherol) levels and the absolute value of the integral from the reflectance spectrum, between 450 and 510 nm, from fat samples were used in a discriminant model, 100% of the lambs were correctly classified according to their feeding system (pasture vs. concentrate).

1. Introduction

Consumers are becoming more health-conscious and there is a trend toward healthy and nutritious foods with more health-promoting functions (Olmedilla-Alonso et al., 2013). In this sense, consumers are increasingly focusing both on the "green image" and the health-promoting properties of products from animals fed on pasture (Dian et al., 2007a; Nozière et al., 2006a; Prache et al., 2003b; Sheath et al., 2001). For these reasons, research on diet authentication in herbivore products is conducted within a general context of increasing consumer awareness concerning the mode of animal production (Dian et al., 2007a). In this regard, certain compounds from the diet, such as carotenoids, have been used to authenticate production system (outdoors vs. indoors) of animal products (Dian et al., 2007b; Prache et al., 2003a; Prache, 2009; Serrano et al., 2007; Sheath et al., 2001). Despite outdoor and indoor production systems, it has been reported (Alcalde et al., 2013; Ripoll et al., 2012) that the most widely used system in small ruminant farming in Mediterranean countries involves suckling kids and lambs, which are slaughtered at low live weights of approximately 10–12 kg. Therefore it seems useful to evaluate the existence or not of correlations between the levels of carotenoids and other micronutrients in the plasma and milk of the ewe and the plasma of the suckling lamb as a possible means to authenticate the exclusive milk diet of these animals.

Mammals are not able to synthesize vitamin A (retinol) and E (mainly in the form of α-tocopherol), so these vitamins must be provided by the diet. Provitamin-A carotenoids (mainly β-carotene) and tocopherol are abundant in fresh pastures and forage, whereas concentrate feedstuffs tend to present very low levels of these compounds, since oxidation may occur during manufacture (Dunne et al., 2009; Nozière et al., 2006a; Ramírez and Quiles, 2005; Schweigert, 1998). Furthermore, the role of fat-soluble vitamins in the nutritional and sensory properties of foods has been recently pointed out (Sauvant et al., 2011), so these vitamins would be significant for consumers not only

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for traceability purposes, but also from the nutritional point of view.

Besides the presence of carotenoids in tissues, other techniques have been developed, in order to discriminate pasture-fed animals from animals with a diet based on concentrate. A mathematical analysis of fat reflectance as an indirect system has been proposed to estimate the concentration of carotenoids (Prache and Theriez, 1999), in order to discriminate two production systems (extensive vs. intensive) in herbivores. This method has been validated in sheep in a large cohort of animals (Dian et al., 2007a). However, the carotenoid concentration in animal tissue can range widely according to dietary supply (Dian et al., 2007b). So, it would be interesting to seek for new compounds and/or methodologies that could be used to differentiate feeding systems individually or in combination with those already developed, in order to increase the degree of discrimination.

In consequence, the aims of this study were to assess the effect of different diets on the carotenoids and fat-soluble vitamin levels of retinol and α-tocopherol in different sheep tissues, and to study the possible links with the livestock production system, for both nutrition and traceability. Another objective was to evaluate if their use in combination with color measurements was useful to discriminate pasture-fed from stall-fed lambs. Finally, the possible existence of correlations between the levels of fat-soluble vitamins and carotenoids of the ewe and the suckling lamb was also studied.

2. Material and methods

2.1. Animals and diets

Three groups of lambs, all from the *Merino* breed, were considered for this study. Group 1 (G1) included 15 male light lambs, which were weaned at 45 days old and reared in an extensive system with a diet based on pasture until the moment of slaughter (around 90 days old), when the animals had an average weight of 21.05 ± 1.02 kg. The paddock where the lambs were reared was in the southwest of Spain, an area characterized by a *dehesa* landscape, i.e. a forest of oaks, cork oaks and other species from different *Quercus spp.*, alongside grassland. Specifically for this paddock the pasture was mainly composed of plants from *Poaceae* family, such as *Agrostis spp.*, *Vulpia spp.*, *Poa spp.*, *Bromus spp.* and *Lollium spp.* Species from *Fabaceae* family were also present; like *Trifolium spp.*, *Ornithopus spp.*, *Medicago spp.* and *Lotus spp.* Animals from group 2 (G2) were also 15 light lambs weaned at 45 days and fattened in an intensive system until slaughter (around 90 days old). These animals had a diet based on concentrates, composed of barley (40.1%), maize (30.0%), wheat (4.0%), soy (22.0%), fat (1.0%), calcium carbonate (1.6%), salt (0.3%), sepiolite (0.5%), flavoring (0.1%) and a corrector for minerals (0.4%). At slaughter these lambs has an average weight of 24.79 ± 0.52 kg. Finally, group 3 (G3) consisted of 15 suckling lambs (around fifteen days old) with an average live weight of 7.18 ± 0.23 kg. Their mothers were grass-fed with a supplementation of oat ad libitum as whole grains. They were reared in a paddock in the southwest of Spain, in a *dehesa* forest too; therefore the main composition of pasture was similar to that in group 1. Water and salt blocks were always available.

2.2. Sampling

Representative samples of the different diets were taken in triplicate from the farms and analyzed. In the case of pasture samples, the sampling areas were randomly selected where the animals were reared. The pasture was sampled from the ground; to do this, three sampling squares (0.5 m by 0.5 m) were randomly established. Finally, pasture samples were characterized overall according to the majority herbaceous species present at genus level. After collecting, all food samples were transported to the laboratory, lyophilized and stored at −80 °C until analysis. In the case of milk samples (G3), 20 mL of milk were collected from each ewe in the second week of lactation. These samples were also transported to the laboratory and stored at −80 °C until analysis.

Lambs from groups 1 and 2 were slaughtered in spring according to the European Regulation (CE No. 1099/2009 of 24 September 2009). Animals arrived at the abattoir by truck the night before slaughter and had access to water until 30 min before slaughtering. The slaughterhouse was 110 km from the pasture paddock (G1) and 74 km from the stall (G2). At the moment of slaughter blood samples were taken from each animal using Li-Heparin as anticoagulant. In addition, in the course of carcass dressing immediately after slaughter, 3 g of adipose tissue from the perirenal area, i.e. from the top of the kidney, of each animal were also collected. This location was selected as previous authors (Priolo et al., 2002) reported that the difference between feeding treatments was significantly higher for perirenal than for subcutaneous caudal fat in lambs, supporting the hypothesis of a greater accumulation of carotenoid pigments in perirenal compared to caudal fat in lambs fed grass. One hour after slaughter, the instrumental color measurements of the perirenal fat samples were carried out. Then, the samples were transported to the laboratory at 4 °C, blood was then centrifuged (1500 × *g*, 10 min, 4 °C), and the plasma was collected. All samples were stored at −80 °C until further analysis.

With respect to suckling lambs from group 3 and their mothers, blood samples were taken at the farm from the jugular vein of each animal, using Li-Heparin as anticoagulant. In this case, blood samples were also transported to the laboratory at 4 °C to centrifuge them (1500 × *g*, 10 min, 4 °C) and collect the plasma, which was stored at −80 °C until analysis. Prior to the analysis, all samples were thawed overnight in the dark at 4 °C.

2.3. Instrumental color measurements

The perirenal fat *CIELAB* (CIE, 1986) color parameters (C^*_{ab}, L^*, a^*, b^* and h_{ab}) were measured on a CM-700d spectrocolorimeter (Konica Minolta Holdings, Inc., Osaka, Japan), using a D_{65} illuminant, the 10° observer and zero and white calibration. Reflectance spectra in the visible region (between 360 and 740 nm, with 10 nm increments) were also acquired and recorded in order to obtain translated reflectance values (TR_i) and the absolute value of the integral (*AVI*) of the translated spectra. In previous studies (Priolo et al., 2002; Ripoll et al., 2008; Zawadzki et al., 2013) the reflectance spectra between 510 and 450 nm were translated to make the reflectance value at 510 nm equal to zero (*TR*). The translated reflectance values (TR_i) were calculated from the reflectance values (R_i) as follows: $TR_i = R_i - R_{510}$, with $I = 360, 370, 380, \ldots, 740$; whereas *AVI* of the translated spectra were calculated according to the following formula;

$$AVI = [(TR_{450}/2) + TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + (TR_{510}/2)] \times 10$$

2.4. Carotenoids, retinol and α-tocopherol extractions

2.4.1. Diets samples

Pasture and concentrate samples were analyzed in triplicate according to the methodology described previously (Kean et al., 2007; Panfili et al., 2004) with some modifications. One gram of each sample was lyophilized prior to the analysis. The extractions were carried out on the lyophilized extracts with a hexane/ethanol mixture (1:1, v/v) as extractant. Two extractions with 3 mL were

carried out. The organic phases obtained were pooled and saponified with 15% ethanolic potassium hydroxide (w/v) overnight at room temperature in the dark and under nitrogen atmosphere. Finally, the organic phase was washed several times with the same volume of water using a pH indicator to know when all the potassium hydroxide was removed. After that, the organic phase was collected and dried using a concentrator (Concentrator plus; Eppendorf Research®, Madrid, Spain). The dry residue was dissolved in 100 μL of ethyl acetate for HPLC analysis.

Extraction of carotenoids and fat-soluble vitamins from milk was carried out using published methodologies (Hulshof et al., 2006; Jewell et al., 2004; Nozière et al., 2006b) with some modifications. First, 1 mL of milk was mixed with 0.5 mL of distilled water, 0.25 mL of ammonia solution 25% (w/v), 1 mL of ethanol and 1 mL of hexane. Samples were then centrifuged (1500 × g, 10 min, 4 °C) and the upper organic phase collected and extracted once more. The organic phases were pooled and 2 mL of sodium hydroxide in ethanol 15% (w/v) were added. The air of the containers was replaced with nitrogen and the saponification reaction was maintained overnight at room temperature in the dark. Finally, the organic phase was washed several times with water, collected and dried (Concentrator plus). The dry extract was dissolved in 35 μL of ethyl acetate for HPLC analysis.

2.4.2. Plasma samples

The methodology described elsewhere (Lyan et al., 2001) was used with some modifications. Plasma (2 mL) diluted with 1 mL of distilled water was deproteinized by adding 2 mL of ethanol. Carotenoids, retinol and α-tocopherol were extracted twice with 2 mL of hexane and the extracts were evaporated to dryness (Concentrator plus). The dry extracts were dissolved in 35 μL of ethyl acetate and injected into the HPLC system.

2.4.3. Fat samples

This extraction was carried out following published methodology (Dunne et al., 2006; Nozière et al., 2006b) with some modifications. Perirenal fat (500 mg) was mixed with 1 mL of 3,5-di-*tert*-4-butylhydroxytoluene (BHT) ethanolic solution (12%, w/v); 2.5 mL of sodium hydroxide in ethanol 30% (w/v) and 5 mL of ethanol were also added. The saponification reaction was carried out overnight at room temperature and in the dark. Water was finally added to stop the reaction and the analytes were extracted with 10 mL of an ether/hexane mixture (2:1, v/v). The samples were then centrifuged (1500 × g, 10 min, 4 °C) and the upper organic phase collected. The extraction was repeated twice. The organic phases were pooled, washed several times with water, collected and dried (Concentrator plus). The residue was dissolved in 1.5 mL of ethyl acetate and filtered for HPLC analysis.

2.5. HPLC analysis

The analyses were performed on an Agilent 1100 system (Agilent, Palo Alto, CA) fitted with a photodiode array detector, a quaternary pump, a column temperature control module set at 20 °C and an autosampler set to draw 20 μL aliquots from the concentrated extracts. A YMC C_{30} column (5 μm, 250 mm × 4.6 mm; YMC, Wilmington, NC) was used. The mobile phase consisted of methanol (MeOH), methyl-*tert*-butyl-ether (MTBE) and water according to the linear gradient; 0 min: 90% MeOH + 5% MTBE + 5% water; 12 min: 95% MeOH + 5% MTBE; 25 min: 89% MeOH + 11% MTBE; 40 min: 75% MeOH + 25% MTBE; 50 min: 40% MeOH + 60% MTBE; 56 min: 15% MeOH + 85% MTBE; 62 min: 90% MeOH + 5% MTBE + 5% water. The mobile phase was pumped at 1 mL/min and the chromatograms were monitored at 450 nm for carotenoids, 325 nm for retinoids and 280 nm for tocopherol. The carotenoids detected were identified by comparison of their spectroscopic and chromatographic characteristics with those of standards obtained from natural sources by recommended procedures as described elsewhere (Meléndez-Martínez et al., 2009). Retinoids and α-tocopherol were identified by using standards from Sigma Chemical (Madrid, Spain). The quantification was performed using calibration curves of standard solutions.

2.6. Statistical analysis

SPSS 15.0 software for Windows (2006; SPSS Inc., Chicago, IL) was used. Analysis of variance (ANOVA) was applied to assess the existence of significant differences. The significant differences between group means were determined by a Tukey post hoc test, with a significance level of $p < 0.05$. A generalized linear model (GLM) was used to study the effect of both weight and age of the animals on the levels of carotenoids, retinol and tocopherol in plasma. In addition, correlations analyses were performed between retinol levels in the milk and plasma of the ewes and the plasma of their suckling lambs using the GLM procedure of SPSS. Finally, two discriminant analyses (DA) were carried out using a stepwise model considering the diet of the animals as an independent variable. The discriminant classification method was leave-one-out cross-validation. The first DA was based on retinol and α-tocopherol plasma levels and the second one on the retinol and α-tocopherol levels and *AVI* values in the adipose tissue. The significance level for a variable to be included in the model was 0.05.

3. Results and discussion

3.1. Carotenoid and vitamins levels in the diets

Table 1 summarizes the information relative to the main carotenoids found in the diet of the animals used in this study. As it can be observed in the table, pasture used in the diet of light lambs (G1) and that used for the mothers of the suckling lambs (G3) showed a carotenoid profile quite similar, since just violaxanthin content showed significant differences ($p < 0.001$) between both groups. Furthermore, for both groups, the main carotenoids found in pasture were lutein (37.3% of total carotenoids in group 1 and

Table 1
Mean concentrations (μg/g DM), standard error, ANOVA and multiple comparison test Tukey-*b* for α-tocopherol and the carotenoids identified in the different diets: G1 (light lambs fed on pasture), G2 (lights lambs fed on concentrate) and G3 (lactating ewes of the suckling lambs fed on pasture + oat supplementation).

Compound	Pasture (G1)	Concentrate (G2)	Pasture (G3)	Oat (G3)	*p*-Value
α-Tocopherol	15.6 ± 7.91	n.d.	12.01 ± 1.94	2.80 ± 0.14	0.426
Violaxanthin	2.30^{a} ± 0.48	n.d.	9.87^{b} ± 1.58	0.16^{a} ± 0.09	<0.001
Lutein	48.5^{ab} ± 8.84	0.01^{a} ± 0.008	92.7^{b} ± 18.68	1.90^{a} ± 1.14	0.002
Zeaxanthin	2.97^{ab} ± 0.61	n.d.	6.31^{b} ± 1.04	0.26^{a} ± 0.08	0.005
Antheraxanthin	2.38 ± 0.39	n.d.	1.48 ± 0.34	n.d.	0.146
β-Carotene	74.1^{ab} ± 15.0	n.d.	61.1^{b} ± 12.5	1.60^{a} ± 1.27	0.017

Significant differences ($p < 0.05$) within a row are indicated by superscripts (a and b), i.e. variables with different superscripts were significantly different among them and variables with the same superscripts were not significantly different. n.d.: not detected.

54.1% in group 3) and β-carotene (56.9% and 35.6% in groups 1 and 3, respectively). These data are in accordance with studies previously reported (Cardinault et al., 2008; Prache et al., 2009), where lutein and β-carotene were detected as the main carotenoids in fresh pasture. It has been reported (Dunne et al., 2009; Nozière et al., 2006a) that the manufacture of concentrate rations involves the exposure to high temperatures coupled with grinding, mixing with minerals, the addition of fat and pelleting, which may involve the oxidation of carotenoids. Despite all these difficulties, previous studies (Nozière et al., 2006a; Röhrle et al., 2011) described the presence of lutein, zeaxanthin and β-carotene in cereal-based concentrate samples. These results are only partially in agreement with our study (Table 1), as only small amounts of lutein were found for concentrate samples (G2). It can also be observed in Table 1 that the carotenoid profile of concentrate was clearly different from the pasture. With respect to the vitamins, retinol was not detected in pasture nor in oat or concentrate samples, while α-tocopherol was present in all except for the concentrate. The absence of retinol in feedstuff was expected since vitamin A in plants is present as pro-vitamin carotenoids instead of retinol. Furthermore, it has been reported (Ramírez and Quiles, 2005) that vitamin E is widely distributed in nature and it is found in the leaves and other green parts of plants. Moreover, it has been stated (Dunne et al., 2009) that when feeds are pelleted, destruction of both vitamins E and A may occur, if the diet does not contain sufficient antioxidants to prevent their oxidation under conditions of moisture and high temperature. This agrees well with our results, since none of the vitamins was detected in the concentrate samples (G2). Finally, significant differences were not found for α-tocopherol level among the diets studied (Table 1). Considering all these data it can be stated that there were differences in the carotenoid profile, as well as, in α-tocopherol level among the feedstuffs studied. Therefore, it could be expected that the levels of these compounds in animal tissues would show differences for stall-fed animals with respect to pasture-fed animals. This fact, as well as its absence of in concentrate samples, could lead to the assessment that α-tocopherol may be useful to differentiate stall-fed lambs from those with a diet based on pasture.

Finally, no carotenoids were found in milk samples, although retinol was (1.59 ± 1.02 μg/mL milk). This could be due to the fact that the β-carotene absorbed by sheep is thought to be almost entirely transformed into retinol, since intestinal cleavage activity of 15-15′-dioxygenase has been reported to be more efficient in sheep compared to cattle (Cardinault et al., 2006). α-Tocopherol was also found (0.97 ± 0.40 μg/mL milk), which was expected due to the presence of this vitamin in the diet of the ewes. Therefore, it could be claimed that milk from ewes on a pasture-based diet would be useful from a nutritional and consumer's point of view because of the presence of both retinol and tocopherol. Furthermore, it was expected that these two fat-soluble vitamins found in milk appeared in the plasma of the suckling lambs and, in this way, help to differentiate their plasma from that of the other lambs, as well as to confirm the diet based exclusively on milk.

3.2. Carotenoid and vitamins levels in plasma

It has been established (Yang et al., 1992) that no β-carotene was detected in the serum of sheep, but small amounts of lutein were present. In this sense, other authors (Dian et al., 2007a,b; Prache et al., 2003a) established a crude estimation of total plasma carotenoid concentration (PCC) for lambs by spectrophotometry considering the concentration obtained as lutein concentration. Nevertheless, both lutein and β-carotene were found in the plasma of the light lambs fed on pasture in the present study (Table 2), being to our knowledge, the first time that β-carotene has been detected in the plasma of lambs (Fig. 1). The rest of the animals studied did not show lutein nor β-carotene in their plasma (Table 2). The absence of these compounds in the stall-fed light lambs was expected since only lutein (in a very low concentration) was observed (Table 1) in their diet. These results are partially in agreement with other authors (Dian et al., 2007a), who reported very low values for PCC in stall-fed lambs. Moreover, since their mothers did not show detectable carotenoid levels in their milk, they were not expected to be detected in the plasma of the suckling lambs (Table 2). Considering these results, lutein and β-carotene could be considered as a useful means to help differentiate animals fed on pasture from the other two diets considered, i.e. concentrate and milk.

On the other hand, retinol and α-tocopherol were found in the plasma of all animals (Table 2). Animals from G2 showed α-tocopherol in their plasma despite its absence in their diet. This may be attributed to supplementation of the concentrate rations with this vitamin before the start of this trial. On the other hand, the plasma levels of α-tocopherol were much higher (ca. 20-to 56-fold) than those of retinol. Other authors pointed to the fact that this may be due in part to an interaction between α-tocopherol and β-carotene (precursor of retinol) as, in ruminants, high concentrations of one appear to reduce the tissue concentrations of the other. It is known (Yang et al., 2002) that the metabolism of fat-soluble vitamins is dependent upon micelle formation for transport across intestinal membranes where the vitamins are incorporated into lipoproteins and secreted into the intestinal lymph for distribution to other tissues. High concentrations of different fat-soluble vitamins may result in competition in the absorption and transport process. In this sense, it has been established (Yang et al., 2002) that, for plasma from lambs fed on pasture, a high concentration of α-tocopherol may interfere with the absorption and metabolism (i.e. its conversion to retinol) of β-carotene and, as both β-carotene and α-tocopherol are found in lipoproteins when transported in the blood, they may compete with each other for their incorporation in the lipoprotein molecule. In addition, significant differences appeared (Table 2) in the level of retinol ($p < 0.001$) and α-tocopherol ($p < 0.05$), for the plasma of all the groups of animals studied. In addition, a first discriminant analysis based on retinol and α-tocopherol plasma levels was carried out (Table 3), in order to explore the utility of these two vitamins to discriminate between the feeding systems.

Table 2
Mean concentrations (μg/100 mL plasma), standard error, ANOVA, Tukey multiple comparison test and effect of weight (*W*) and age (*A*) of animals on retinol, lutein, β-carotene and α-tocopherol in the plasma of the three groups of animals: G1 (light lambs fed on pasture), G2 (light lambs fed on concentrate), G3 (suckling lambs).

Compound	G1	G2	G3	*p*-Value group	*p*-Value weight	*p*-Value age	*p*-Value *W***A*
Retinol	$2.81^{a} \pm 0.27$	$4.04^{b} \pm 0.53$	$2.49^{a} \pm 0.20$	0.011	0.177	0.994	0.609
α-Tocopherol	$158^{b} \pm 18.8$	$123^{ab} \pm 21.8$	$86.3^{a} \pm 17.4$	0.043	0.628	0.258	0.328
β-Carotene	2.97 ± 0.68	n.d.	n.d.	–	–	–	–
Lutein	4.95 ± 0.71	n.d.	n.d.	–	–	–	–

Significant differences ($p < 0.05$) within a column are indicated by superscripts (a and b), i.e. variables with different superscripts were significantly different among them and variables with the same superscripts were not significantly different; n.d.: not detected. 15 animals per group.

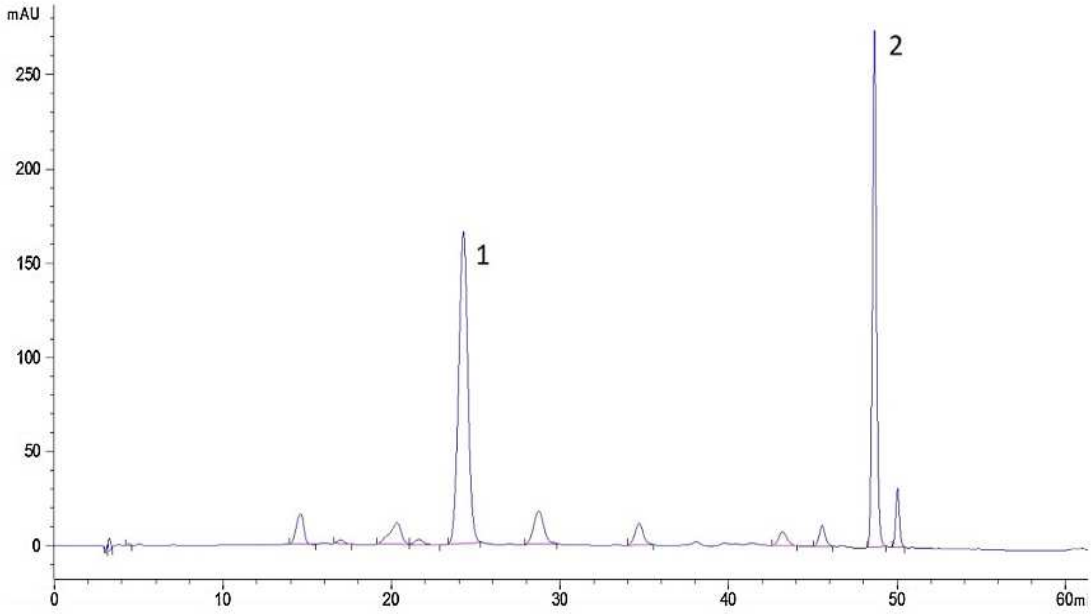

Fig. 1. Typical HPLC chromatogram of the plasma of lambs fed on pasture. Peak 1 corresponds to lutein and peak 2 to β-carotene.

In this way, 75.0% of light lambs fed on pasture, 53.3% of light lambs with a diet based on concentrate and 60.0% of suckling lambs were correctly classified according to their diet, i.e. 61.9% of all the animals were correctly classified into their group. According to these data, it could be stated that only retinol and α-tocopherol in plasma cannot completely differentiate lambs according to their diet.

Finally, as the milk was the only feed of the suckling lambs (G3) it could be expected that the levels of retinol and α-tocopherol in their blood system were correlated with the levels in the system of the mothers, but significant correlations were not found. These results may be due, among other factors, to a high inter-individual variability in the absorption, circulation and storage of these vitamins. In this sense, previous studies (Dian et al., 2007a,b; Prache et al., 2003b) have confirmed a high inter-individual variability in plasma and fat carotenoid and fat-soluble vitamins concentrations, probably due to inter-individual variations in intake levels, as well as, in absorption and metabolism of these compounds. Therefore, more studies are needed in this sense.

3.3. Carotenoid and vitamin levels in fat

Previous studies (Prache et al., 2003a; Yang et al., 1992) have reported lutein as the only carotenoid stored in the fat of sheep. Indeed, the usefulness of carotenoids concentration in fat of sheep to authenticate pasture feeding system has been stated (Dian et al., 2007b). However, to our knowledge, neither retinol nor α-tocopherol have been proposed previously for sheep with this aim, although they have been used in this sense in cattle (Calderón et al., 2007; Nozière et al., 2006a). In Table 4 the mean values and analysis of variance for retinol and α-tocopherol in the fat are presented. Carotenoid levels are not shown since they were not detected in any fat sample. This absence of carotenoids is in disagreement with previous studies (Yang et al., 2002), since these authors reported that lutein was present in the adipose tissue of sheep, although they also observed that the amount of this compound was extremely low and negligible compared with the carotenoids present in cattle. Both retinol and α-tocopherol were detected in the fat samples of the two groups of animals studied. The levels of α-tocopherol were higher than those of retinol in both groups, which was expected as one of the main storage sites of tocopherol is the adipose tissue, while for retinol it is the liver (Ortega et al., 2005; Ramírez and Quiles, 2005).

Moreover, there were significant differences for both vitamins ($p < 0.01$ for retinol and $p < 0.05$ for α-tocopherol) between the two groups of lambs (Table 4). In this sense, the levels were always higher for the animals fed in an extensive system than those reared in an intensive system, which would imply that products from sheep fed on pasture have higher amounts of antioxidant fat-soluble vitamins, i.e. retinol and α-tocopherol, improving their value for human nutrition. What is more, it is sensible to infer that

Table 3
Classification matrix resulting from the discriminant analysis based on retinol and α-tocopherol plasma levels: assigned (columns) against real data (rows).

Group	Predicted group (%)		
	Light lambs pasture	Light lambs concentrate	Suckling lambs
1 (light lambs pasture)	**75.0**	8.3	16.7
2 (light lambs concentrate)	13.3	**53.3**	33.3
3 (suckling lambs)	20.0	20.0	**60.0**

15 animals per group.

Table 4
Mean values, standard error and ANOVA for the retinol and α-tocopherol levels in fat (μg/g fat), color parameters (L^*, a^*, b^*, C^* and h_{ab}) and absolute value of the integral of the translated spectra between 450 and 510 nm (*AVI*) measured in the fat of light lambs fed on pasture (group 1) and stall-fed light lambs (group 2).

Variables	Light lambs pasture	Light lambs concentrate	*p*-Value
Retinol	1.48 ± 0.08	1.03 ± 0.08	0.001
α-Tocopherol	42.4 ± 1.76	34.8 ± 0.23	0.031
L^*	81.4 ± 1.16	79.7 ± 0.97	0.285
a^*	2.39 ± 0.75	1.17 ± 0.32	0.120
b^*	14.7 ± 0.78	14.0 ± 0.47	0.430
C^*	15.1 ± 0.85	14.1 ± 0.47	0.292
h_{ab}	81.6 ± 2.59	85.4 ± 1.31	0.179
AVI	262 ± 24.0	126 ± 15.0	<0.001

15 animals per group.

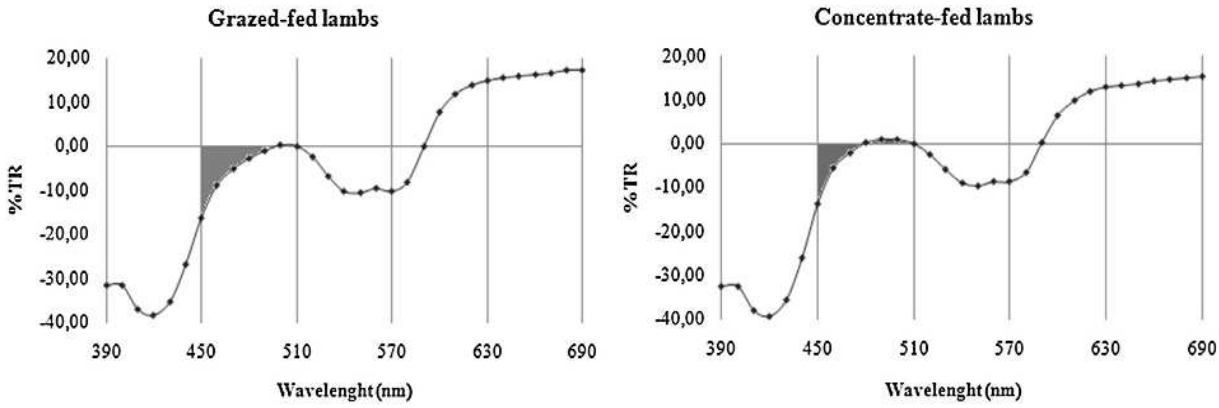

Fig. 2. Reflectance spectra pattern of perirenal fat for light lambs reared on an extensive system with a diet based on pasture (left) and on an intensive system with a diet based on concentrate (right). The reflectance values have been translated to have reflectance at 510 nm equal to zero. The absolute value of the integral (*AVI*) is the shadow are comprised between the curve and *X*-axis in the zone 450–510 nm.

the higher levels of retinol were due to the higher level of provitamin A carotenoids in the pasture, supporting the hypothesis that retinol level in fat can be used as a tool to differentiate lambs fed on pasture. However, it is not clear if the use of α-tocopherol levels in fat is suitable for this purpose. This compound was not detected in the concentrate samples from group 2 but it was present in plasma of lambs from such group. As suggested above, the presence of α-tocopherol in fat could be actually due to a supplementation previous to this trial. Therefore, more studies are needed to confirm the usefulness of α-tocopherol in fat to differentiate lambs according to their feeding system.

3.4. Color and reflectance spectra of the fat

The color parameters (L^*, a^*, b^*, C^*_{ab} and h_{ab}) of the perirenal fat are summarized in Table 4. No significant differences were observed as a function of the animal feeding system. Thus, these parameters did not appear as useful to differentiate lambs according to their diet. Nevertheless, when the percentage of translated reflectance spectrum in the vision region (i.e. from 390 to 690 nm) of the perirenal fat was studied, it was observed significant differences ($p < 0.001$) in the region between 450 and 590 nm, i.e. in the region where carotenoids absorb light, for both types of feeding system. Besides, Fig. 2 shows the average reflectance spectra of the perirenal fat of pasture-fed lambs and concentrate-fed lambs. In these figures it could be observed, according to previous studies (Dian et al., 2007a; Priolo et al., 2002), that animals fed on pasture showed lower absorbances throughout the full visible spectrum than those fed on concentrate. The usefulness of the translated fat spectrum to differentiate between feeding systems (grazing vs. concentrate) in lambs has been established (Ripoll et al., 2008), which would be also possible in the present study due to the significant differences reported above. Moreover, in previous studies (Dian et al., 2007a; Prache et al., 2003a; Priolo et al., 2002) the absolute value of the integral (*AVI*) of the translated spectra between 450 and 510 nm was proposed as a biomarker of feeding traceability in lambs. In this study, the mean value (the integral values were always negative and for this reason is presented as absolute value) of this *AVI* (Table 4) was significantly different ($p < 0.001$) between lambs fed on an extensive system and those fed on an intensive system. These results are in accordance with those reported previously by Priolo et al. (2002), who confirmed higher *AVI* values for grass-than for stall-fed lambs.

Likewise, a positive correlation between plasma carotenoid concentration at slaughter and *AVI* has been established (Prache et al., 2003a; Serrano et al., 2007). Hence the combined use of both plasma carotenoid concentration at slaughter and *AVI* has been proposed (Prache et al., 2003a) as a more precise assessment for the discrimination of lamb production systems. However, and according to the results present above, the detection of retinol and α-tocopherol in fat of both groups of lambs was more suitable than the detection of carotenoids. These statements combined with the fact that the persistence of carotenoids and fat-soluble vitamins is longer in the fat than in the blood (Serrano et al., 2007), led us to carry out a second discriminant analysis in order to assess the utility of the parameters measured in the perirenal fat (Table 4), to differentiate the two feeding systems in lambs. Retinol, α-tocopherol level and the *AVI* were considered in the model. This model allowed correct classification of 100% of the animals according to their diet (pasture vs. concentrate). Thus, it is noteworthy that the combination of these three parameters improves, under our trial conditions, the reliability of the discrimination between feeding systems for lambs.

4. Conclusions

Milk from ewes on a pasture-based diet seemed useful from a nutritional and consumer's point of view because of the presence of the antioxidant vitamins retinol and α-tocopherol. In addition, the presence of lutein and β-carotene in plasma appeared, under our experimental conditions, as a useful tool to discriminate lambs fed exclusively with pasture. More studies are needed to establish the existence or not of correlations in retinol and tocopherol levels between ewe's milk and the plasma of the suckling lambs. Furthermore, the products from sheep fed on pasture would have higher amounts of retinol and α-tocopherol, which could improve their value for human nutrition. Finally, the main result of this trial is that the reliability of the discrimination of lambs according to their feeding system (i.e. pasture vs. concentrate) was improved (100% of the animals accurately classified) when retinol, α-tocopherol and the *AVI* were considered in the discriminant model, providing a useful tool for traceability studies.

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Chapter 3 *Effect of different carotenoid-containing diets on the vitamin A levels and colour parameters in Iberian pigs' tissues: utility as biomarkers of traceability*

ANTECEDENTES

Los consumidores son cada vez más exigentes y reclaman alimentos seguros y de alta calidad, de ahí que el término "trazabilidad" haya ido adquiriendo importancia en los últimos años (Storøy et al, 2013; Van Dijk et al., 2008). La trazabilidad en productos de origen animal puede estar basada en el análisis de micronutrientes que están presentes en la dieta y pueden aparecer como tales o metabolizado en los tejidos de los animales, como los carotenoides o el retinol (Ballin, 2010). Por otra parte, estudios previos (Priolo et al, 2002; Ripoll et al, 2008) informaron que las coordenadas tricromáticas del espacio CIELab fueron útiles para obtener el 100% de discriminación entre corderos alimentados con forraje o con alimentos concentrados. Sin embargo, pocos estudios se han realizado en ganado porcino (Carrapiso y García, 2005).

El cerdo ibérico es uno de los más importantes en área Mediterránea, tanto en el tamaño de la población como en su importancia económica (Juárez et al., 2009). De acuerdo con la nueva legislación española (BOE, 2014) existen tres categorías diferentes para los productos del cerdo ibérico según su sistema de alimentación. Los productos de alta calidad están relacionados con el sistema de *Montanera*, con una dieta a base de bellotas y pastos. Por lo tanto, sería interesante poder diferenciar los productos procedentes de animales criados en *Montanera* de las otras estrategias de crianza de cerdos ibéricos.

OBJETIVOS

El objetivo principal de este estudio fue buscar nuevas herramientas para diferenciar los productos del cerdo ibérico de acuerdo con el sistema de cría de los cerdos. En este sentido, también se propusieron los siguientes objetivos específicos:

- Evaluar el efecto de tres dietas con distinto contenido en carotenoides en los niveles de vitamina A en los tejidos de cerdo ibérico y el color de la grasa perirrenal.
- Evaluar la utilidad de los niveles de retinoides en diferentes tejidos de cerdo ibérico como una herramienta de trazabilidad para diferenciar los sistemas de cría de estos animales.

DISEÑO EXPERIMENTAL

Para este estudio se dividieron treinta cerdos ibéricos en dos grupos según su sistema de producción: *Montanera* (basada en bellotas y pasto) y *Cebo* (dieta basada en alimentos concentrados). Se recogieron muestras representativas de los alimentos de los animales de cada explotación. Además, se recogieron, en el momento del sacrificio, muestras de sangre, grasa perirenal e hígado de cada animal.

Se midió el color (C^*_{ab}, L*, a*, b* y h_{ab}) de la grasa perirenal siguiendo la metodología CIELab. También se calcularon, de acuerdo a la metodología descrita por autores previos (Prache & Theriez, 1999; Priolo et al., 2002; Ripoll et al., 2008; Zawadzki et al., 2013), los valores de los espectros de reflectancia trasladados (TR, de las siglas en inglés) así como el Valor Absoluto de la Integral (AVI, de sus siglas en inglés).

La extracción de carotenoides de los alimentos se realizó de acuerdo a métodos publicados por otros autores (Kean et al., 2007; Panfili et al., 2004). Las extracciones en las muestras de plasma y grasa perirenal se hicieron siguiendo la metodología descrita

por Lyan et al. (2001) Dunne et al. (2006), respectivamente. Finalmente, para las extracciones de retinoides y carotenoides en los hígados se tuvo en cuenta el método analítico publicado por Woodall et al. (1996). El contenido en carotenoides y retinoides de todas las muestras fue analizado por HPLC y la identificación de los ésteres de retinol en hígado se realizó mediante análisis HPLC-MS.

RESULTADOS

La dieta de los animales criados en un sistema de *Montanera* fue significativamente diferente ($P<0.01$), en cuanto al perfil en carotenoides, de la dieta del grupo criado en un sistema de *Cebo*, por lo tanto cabría esperar que el contenido en carotenoides y retinoides en los tejidos de los dos grupos de animales fueses también diferentes. Sin embargo, no se detectaron carotenoides en ninguno de los tejidos biológicos de cerdo ibérico estudiados, aunque sí se detectaron retinoides.

Cuatro ésteres de retinol, además de (todo-*trans*)-retinol, fueron identificados por HPLC-MS en el hígado de cerdos ibéricos. La forma predominante de la vitamina A en hígado fue el palmitato de retinol: 45.8% de la vitamina A en el hígado de los cerdos criados en Montanera y 44.1% de aquellos criados en un sistema de Cebo, siendo significativamente mayor en el primer grupo ($P<0.05$). El contenido tanto de (todo-*t*)-retinol como de linoleato de retinol en hígado fue, también, mayor ($P<0.05$) para los animales alimentados en un sistema de *Montanera*. Por último, no se encontraron diferencias significativas entre los dos grupos de cerdos ibéricos ni para el oleato de retinol ni para el estearato de retinol.

Tampoco se encontraron diferencias significativas entre los dos grupos respecto a la concentración de retinol en plasma y grasa perirenal. Sin embargo, sí hubo diferencias ($P<0.01$) en el parámetro de color L* en la grasa perirenal según la alimentación de los

animales, aunque el resto de los parámetros de color (a*, b*, C^*_{ab}, h_{ab}) no mostraron diferencias en este sentido. Además, ni TR (450-510 nm) ni AVI fueron diferentes entre los dos grupos de cerdos, probablemente debido a la ausencia de carotenoides en la grasa de los animales.

on el fin de evaluar la utilidad de los parámetros estudiados para discriminar los sistemas de alimentación del cerdo ibérico, se realizó un análisis discriminante teniendo en cuenta, por una parte los niveles de retinoides en hígado y por otra los parámetros de color en la grasa perirrenal. En el modelo fueron incluidos los niveles de ésteres de retinol y (todo-*t*)-retinol en el hígado, lo que permitió una correcta clasificación del 92.9% de los animales. Por otro lado, cuando se consideraron los parámetros de color (L* y h_{ab}) en el modelo la clasificación de los animales fue peor, ya que este modelo permitió clasificar correctamente el 78,6% de los animales de acuerdo a su sistema de cría.

CONCLUSIONES

De acuerdo con los resultados obtenidos en este estudio, las principales conclusiones son:

1. El contenido en retinol en plasma y grasa perirenal de cerdo ibérico no se vio afectado por la dieta de los animales.
2. El contenido en retinoides en el hígado de los cerdos ibéricos se mostró como una herramienta útil para diferenciar estos animales según su dieta y, por tanto, para ser usada en estudios de trazabilidad.
3. Algunos parámetros de color (L * y h_{ab}) en la grasa perirrenal parecieron, también, útiles para diferenciar los cerdos ibéricos de acuerdo a su dieta.

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Effect of different carotenoid-containing diets on the vitamin A levels and colour parameters in Iberian pigs' tissues: utility as biomarkers of traceability

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ABSTRACT

Retinol and fat colour parameters in Iberian pigs fed on different carotenoid-containing diets were assessed. Thirty animals in two groups were considered: Iberian breed pigs fed on acorns and grass (*Montanera*) and on concentrate (*Cebo*). Carotenoids and retinoids were analysed in the diets and in plasma, liver and perirenal fat of the animals by HPLC and HPLC-MS. Retinol levels in plasma and fat were similar in *Montanera* and *Cebo* animals. The utility of retonids and colour parameters as traceability index was also explored. Retinoids in liver classified correctly 93% of the animals according to their diet L^* and h_{ab}. CIELAB parameters of the perirenal fat discriminated correctly 78.6% of the animals according to their diet. L^* values for the *Montanera* animals were significantly different ($P < 0.01$) from those fed on concentrate. It can be claimed that the liver retinol profile and fat colour parameters can be useful for feeding traceability purposes in Iberian pigs breed in *Montanera* and *Cebo*.

1. Introduction

Nowadays, food trade is one of the largest global businesses and there has been a recent necessity to implement an open and transparent communication with consumers about food safety, policy procedures and decision making practices. Furthermore, consumers all over the world are increasingly demanding and claiming safe and high quality food products. Thus, the term "traceability" has been acquiring importance over the last few years (Storoy, Thakur, & Olsen, 2013; Van Dijk et al., 2008). In this sense, traceability is defined by the international standard ISO 8402 as "the ability to trace the history, application or location of an entity by means of recorded identifications" (ISO, 1994).

Traceability in animal products can be based on analysis of different chemical constituents that are present in the feedstuffs (e.g. pasture, hay, maize, and concentrate), and can appear as such or metabolized in the animals' blood and tissues upon consumption (Ballin, 2010). In this sense, carotenoids have been proposed as biomarkers of traceability in order to differentiate grazing production systems in herbivores. Their presence in some tissues is attributed to their ingestion with the diet since it has been demonstrated (Prache, Priolo, & Grolier, 2003) that animals are unable to synthesize them *de novo* endogenously. Besides, it is known that the content of these pigments in adipose tissue may be responsible for differences in carcass fat colour since these pigments generate yellow (lutein), orange (β-carotene) and red colours (Ripoll, Joy, Muñoz, & Albertí, 2008). Moreover, Ripoll, Joy, Muñoz, Albertí, and Delfa (2006) reported that the trichromatic coordinates of the CIELAB space were useful to obtain 100% discrimination between forage and concentrate-fed lambs. However, few studies have been conducted in pigs (Carrapiso & García, 2005).

On the other hand, one of the main function of some carotenoids in mammals is to be precursors of vitamin A (i.e. retinol and retinyl esters) (Schweigert, 1998), which is an essential dietary factor for cell growth and differentiation, reproduction, maintenance of immune system, and vision (Ball, 2006). The vitamin A activity is exhibited by those carotenoids which possess, at least, an unsubstituted β-ring in their structure as well as an appropriate unsaturated backbone (such as β-carotene, α-carotene and β-cryptoxanthin) (Nozière et al., 2006). This transformation is catalysed by the cleavage of the carotenoids (mainly at the intestine) by the enzymeβ-carotene 15,15′-dioxygenase (Nagao, During, Hoshino, Terao, & Olson, 1996). Furthermore, pigs, as well as other animals like goats, are known not to absorb carotenoids at all or at least only at very low levels (Schweigert, 1998), but they can cleave provitamin A carotenoids into retinoids. Different carotenoid-containing

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diets could, therefore, result in differences in the levels of retinol in the tissues, as well as on the fat colour parameters. Scarce information can be found in the literature on this subject in relation to Iberian pigs.

The Iberian pig breed is one of the most important Mediterranean swine type, both in population size and economic importance (Juárez, Clemente, Polvillo, & Molina, 2009). It is an autochthonous pig breed from the Iberian Peninsula, which is heavier with thicker fat depots than current industrial breeds, and produces higher quality derived products (Gosálvez et al., 2007). According to the new Spanish legislation related to the quality standard for Iberian pig products (BOE, 2014) three different categories are considered based on feeding and rearing systems. The highest quality products are related to the typical outdoor rearing system with a nutritional strategy based on acorns and grass (*Montanera*) while the animals fed on concentrate can be reared in an extensive (*Cebo de campo*) or in an intensive indoor system (*Cebo*). The *Montanera* rearing system is associated with an increase in animal welfare, reduced environmental impact and protection of a traditional production system. It is carried out in the dehesa, an agroforestry system which is a combination of grazing, woodland and cropping lands. This system predominates in the Western and Southern lowlands of the Iberian Peninsula where the Iberian pig production has vital importance (Gaspar, Mesías, Escribano, Rodriguez De Ledesma, & Pulido, 2007; Jurado et al., 2013; López-Bote et al., 2008).

Taking all these points into consideration, the aim of this study was to assess the effect that different carotenoid-containing diets have on the vitamin A levels and colour parameters in Iberian pigs' tissues. Also the utility of retinoids in different tissues as well as the perirenal fat colour was explored as a feeding traceability tool for different quality products in Iberian pigs.

2. Material and methods

2.1. Animals and diets

Thirty animals divided into two groups (2 × 15) were considered for this study. Animals in group Montanera (M) were 14 month-old 100% Iberian breed pigs and had, at the moment of the slaughter, an average weight of 146 ± 3 kg. They were reared in a *Montanera* feeding system, based on acorns from different *Quercus spp.* and pastures, mainly from *Arbutus spp.*, *Thymus spp.*, *Cistus spp.* and *Erica spp.* In this extensive system the pigs have a higher physical activity. Those in group Cebo (C) were also 14 month-old 100% Iberian breed pigs with an average weight of 168 ± 7 kg; they were reared indoor with a diet based on concentrate containing corn, wheat, barley, alfalfa, beet pulp, soy and sunflower cake. None of animals' diet was Vitamin A supplemented, as checked with the producers. All animals were slaughtered at commercial weights in Spain, in an abattoir in Southwest of Spain according to the European Regulations (Council Regulation (EC) Nº 1099/2009 of 24 September, 2009).

2.2. Sampling

Representative samples of the diets were taken from the farms and analysed. In the case of the group M, the sampling areas were randomly selected at the dehesa where the animals were reared (in Southwest Spain). The acorn samples, which had dropped from randomly selected trees, were collected directly from the ground. The sampling area corresponded to those locations where the pigs were actually found during their rearing. The pasture was also sampled from the ground; to do this six fixed sampling quadrates (0.5 m by 1.0 m) were randomly established within each plot. Finally, pasture samples were characterized overall according to the majority herbaceous species present at the gender level (Tejerina, García-Torres, Cabeza de Vaca, Vázquez, & Cava, 2011). After collecting, samples were transported to the laboratory and, then, they were stored at −80 °C until analysis. Five grams of sample was used for the analysis.

Ten millilitres (mL) of blood was taken from each animal at the moment of slaughter using Li-heparin as anticoagulant. The blood samples were transported to the laboratory at 4 °C and then centrifuged (8000 *g*, 10 min, 4 °C); the plasma collected was stored at −80 °C until analysis. In addition, also at the moment of the slaughter, 2 g of liver and 3 g of fat from the perirenal area of the carcass were collected. One hour after the slaughter, the instrumental colour measurements of the perirenal fat samples were carried out. Finally, the liver and fat samples were stored at −80 °C until further analyses. Prior to the analysis, all the samples were unfrozen overnight and in the dark in a fridge at 4 °C.

2.3. Instrumental colour measurement

The perirenal fat CIELAB (CIE, 1986) colour parameters (C_{ab}^*, L^*, a^*, b^* and h_{ab}) were measured on a CM-700d spectrocolorimeter (Konica Minolta Holdings, Inc, Osaka, Japan), using the D_{65} Illuminant, the 10° Observer and zero and white calibration. Besides, the reflectance spectra in the visible region (approx. between 360 and 740 nm, considering 10 nm increments) were also acquired and recorded to obtain translated reflectance values (TR_i) and the absolute value of the integral of the translated spectrum (AVI). In previous studies (Priolo, Prache, Micol, & Agabriel, 2002; Ripoll et al., 2008; Zawadzki, Do Prado, & Prache, 2013) the reflectance spectra between 510 and 450 nm were translated to make the reflectance value at 510 nm equal to zero (TR). The translated reflectance values (TR_i) were calculated from the reflectance values (R_i) as follows: $TR_i = R_i - R_{510}$, with i = 360, 370, 380…740, whereas the absolute value of the integral of the translated spectrum (AVI) was calculated according to the following formula:

$$AVI = [(TR_{450}/2) + (TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + TR_{510}/2)] \times 10$$

2.4. Carotenoid and retinol extraction

2.4.1. Feedstuff samples

The feed samples were analysed by a methodology described elsewhere (Kean, Ejeta, Hamaker, & Ferruzzi, 2007; Panfili, Fratianni, & Irano, 2004) with some modifications. The extractions were carried out with a mixture hexane/ethanol (1:1, *v*/*v*). The organic phase obtained was saponified with 15% ethanolic potassium hydroxide (*w*/*v*) overnight at room temperature in the dark and under a nitrogen atmosphere. Finally, the organic phase was washed several times with water, collected and dried using a concentrator (concentrator plus, Eppendorf Research®, Madrid, Spain). The residue was dissolved in 60 μL of ethyl acetate for HPLC analysis.

2.4.2. Plasma samples

The methodology described by Lyan et al. (2001) was used with some modifications. 4 mL of plasma diluted with the same volume of distilled water was deproteinized by adding 4 mL of ethanol. Carotenoids and retinol were extracted twice with 4 mL of hexane and the extracts were evaporated to dryness using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). They were eventually re-dissolved in 35 μL of ethyl acetate and injected on the HPLC system.

2.4.3. Liver samples

In the case of liver sample extractions, the methodology described by (Woodall, Britton, & Jackson, 1996) was followed with some modifications. 0.1 g of liver was lyophilized. Then, samples were homogenized with 600 μL of NaCl water solution 85% (*w*/*v*). Carotenoids and retinoids were extracted with 1 mL dichloromethane. Samples were then centrifuged (8000 *g*, 5 min, 4 °C) and the lower organic phase was collected. The extraction was repeated twice. The organic phases were pooled and dried using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The residue was finally re-dissolved in 35 μL of ethyl acetate for HPLC analysis.

Table 1
Mean concentrations (mg/100 g), standard deviation and ANOVA for the carotenoids identified in the diet of group M (Iberian pigs fed on acorns and pasture) and group C (Iberian pigs fed on concentrate).

Carotenoid	Pasture	Acorn	Concentrate
Violaxanthin	71.70 ± 9.99	n.d.	n.d.
Zeaxanthin	n.d.	n.d.	5.58 ± 0.31
Lutein	169.79 ± 20.64	n.d.	9.53 ± 0.57**
β-carotene	242.93 ± 31.75	n.d.	n.d.
(9Z)-β-carotene	36.15 ± 4.39	n.d.	n.d.

Sig.: significant differences. ** $p < 0.01$, n.s.: not significant.

2.4.4. Fat samples

This extraction was carried out following some methodology previously described (Dunne, O'Mara, Monahan, & Moloney, 2006) with some modifications. 500 mg of perirenal fat was mixed with 1 mL of 3,5-di-tert-4-butylhydroxytoluene (BHT) solution (12% *w*/*v*) in order to suppress oxidation of carotenoids and retinoids. 2.5 mL of sodium hydroxide in ethanol 30% (*w*/*v*) and 5 mL of ethanol were also added. The saponification reaction was carried out overnight at room temperature and in the dark. 3 mL of water was finally added to stop it and the extraction was carried out with 10 mL of a mixture eter/hexane (2:1, *v*/*v*). Samples were then centrifuged (8000 *g*, 10 min, 4 °C) and the upper organic phase was collected. Extraction was repeated twice. The organic phases were pooled, washed several times with water, collected and dried using a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain). The residue was dissolved in 2 mL of ethyl acetate and filtered for chromatographic analysis.

2.5. High performance liquid chromatography (HPLC)

The analyses were performed on an Agilent 1100 system (Agilent, Palo Alto, CA, United States) fitted with a photodiode array detector, a quaternary pump, a column temperature control module set at 20 °C and an auto sampler set to draw 20 μL aliquots from the concentrated extracts. An YMC C_{30} column and an YMC C_{18} column (5 μm, 250 × 4.6 mm) (YMC, Wilmington, NC, USA) were used for the feedstuffs and for the animals' tissues, respectively. The mobile phase consisted on methanol (MeOH), methyl-ter-butyl ether (MTBE) and water according to the linear gradient: 0 min: 90% MeOH + 5% MTBE + 5% water; 12 min: 95% MeOH + 5% MTBE; 25 min: 89% MeOH + 11% MTBE; 40 min: 75% MeOH + 25% MTBE; 50 min: 40% MeOH + 60% MTBE; 56 min: 15% MeOH + 85% MTBE; and 62 min: 90% MeOH + 5% MTBE + 5% water. The mobile phase was pumped at 1 mL/min and the chromatograms were monitored at 450 nm for carotenoids and at 325 nm for retinoids. The carotenoids detected were identified by comparison of their spectroscopic and chromatographic characteristics with those of standards obtained from natural sources by recommended procedures as described elsewhere (Meléndez-Martínez, Vicario, & Heredia, 2009). The quantification was performed by using calibration curves of standard solutions.

2.6. Liquid chromatography–electrospray ionization ion trap/time-of-flight mass spectrometry

Identification of retinyl esters was done by HPLC-MS analysis. The liquid chromatograph in the HPLC-ESI/TOF-MS system was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). The HPLC conditions were the same as described above. An YMC C30 column (5 μm, 250 × 4.6 mm) (YMC, Wilmington, NC, USA) was used. A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer electrospray ion source. Mass spectrometry was performed using a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with Q-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode using a scan range from *m*/*z* 50 to 1200. Mass spectra were acquired in MS fullscan. The instrument control was performed using Bruker Daltonics HyStar 3.2.

2.7. Statistical analysis

The SPSS 15.0 for Windows (SPSS Inc., 2006) software was used. Analysis of variance (ANOVA) test was applied to assess the existence of significant differences with a significance level of $p < 0.05$. Discriminant analyses (DA) was carried out using a stepwise model considering the diet of the animals as independent variable. The discriminant classification method was leave-one-out cross-validation. The significance level for a variable to be included in the model was 0.05. Finally, principal component analysis (PCA) and varimax rotations were applied in order to categorize the groups of animals considering 10 variables of fat, liver and plasma. Bartlett's sphericity test and the Kaiser–Meyer–Olkin (KMO) measure for sampling adequacy were applied to test the validity of the sampling (Gaspar et al., 2007).

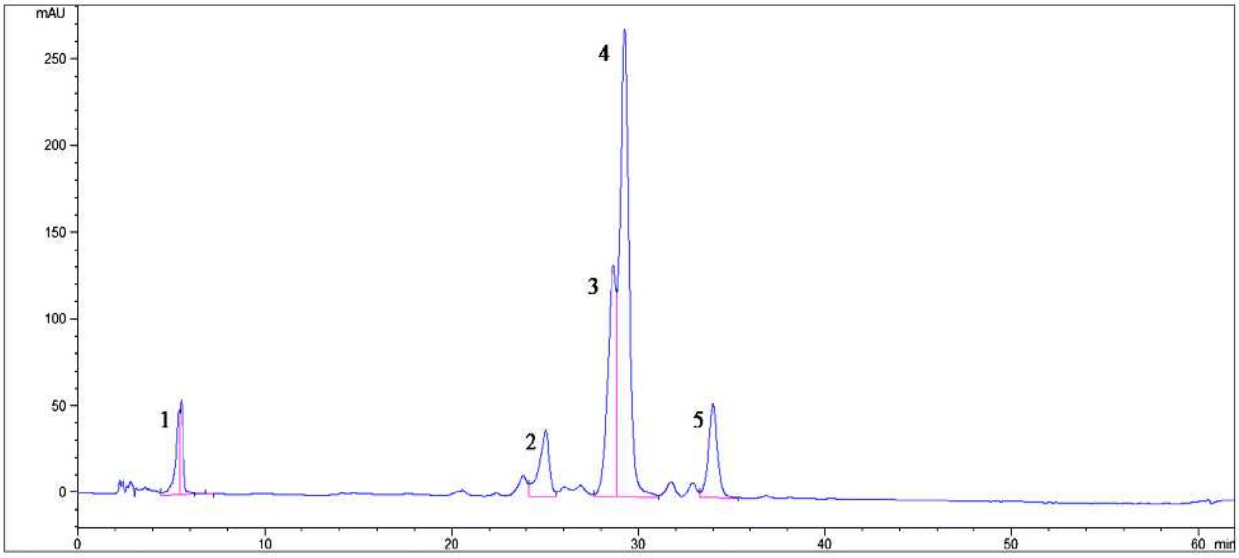

Fig. 1. Typical HPLC chromatogram of the liver retinoid fraction and high accuracy measurements that show the assigned peak of retinoids found in Iberian pig liver.

Table 2
High accuracy measurements that show the assigned formulas of the retinylesters identified in the liver of Iberian pigs.

Peak	R_t (min)	Compound identity	HPLC-PDA spectrum λ_{max} (nm)	Molecular formula	MW. Cal.	MW. meas.	Error (ppm)
1	5.5	All-*t*-retinol	325	$C_{20}H_{30}O$	286.4516	n.a.	n.a.
2	24.8	Retinyllinoleate	325	$C_{38}H_{60}O_2$	549.4868	549.4895	2.7
3	28.6	Retinyloleate	325	$C_{38}H_{62}O_2$	551.5028	551.5057	2.9
4	28.9	Retinylpalmitate	325	$C_{36}H_{60}O_2$	525.4868	525.4638	23.0
5	33.5	Retinyl stearate	325	$C_{38}H_{64}O_2$	553.5188	553.4951	23.7

R_t means retention time. n.a. means not applicable, identified with standard by HPLC-PDA. MW.cal. means molecular weight calculated for $[M + H]^+$. MW.meas. means molecular weight measured for $[M + H]^+$. Error is mean value corresponding to the high accuracy measurements of all isomers of each retinyl ester.

3. Results and discussion

Table 1 summarizes the information relative to the main carotenoids found in the animal feedstuffs analysed in this study. As shown, carotenoids were not detected in the acorn samples. Lutein was the only carotenoid found in all samples, being its concentration significantly higher ($p < 0.01$) in pasture than in concentrate. Both β-carotene and (9Z)-β-carotene were detected in pasture samples. These findings are partially in accordance with previous studies (Cardinault et al., 2008; Prache et al., 2009) where lutein and β-carotene were reported as the main carotenoids in fresh pasture, but (9Z)-β-carotene had not been reported previously. Furthermore, in pasture samples, violaxanthin (Table 1) and (9′Z)-neoxanthin were also detected, which is in agreement with the data reported by Cardinault et al. (2008).

On the other hand, just lutein and zeaxanthin appeared on concentrate from group C (Table 1). These results agree well with the data reported by Nozière et al. (2006), who stated that most concentrated feeds are very low in carotenoids, although maize contains lutein and zeaxanthin, and lower amounts of cryptoxanthin and zeinoxanthin, among other xanthophylls, which are concentrated in corn gluten meal. Overall, from the data obtained in the present study it could be concluded that the diet of the animals fed on *Montanera* was significantly different in terms of its carotenoid profile from the diet in the *Cebo* group, so it would be expectable that the content of carotenoids and retinoids in animals' tissues were also different for both groups.

Fig. 1 shows a typical chromatogram of the retinoid fractions present in the liver of the animals studied. Identification was based on accurate mass, isotopic pattern and fragmentation profile of the molecular ion. Table 2 contains the high accuracy measurements obtained from the ESI mass spectra and the post-processing routine applied with the software. Peak identification was based on the molecular weight of [M + H]+. The mean all-trans-retinol and retinyl esters content in the liver samples as well as the results of ANOVA for the two groups of animals are summarized in Table 3. The predominant form of vitamin A in the livers of both groups of pigs was retinyl palmitate, in accordance with data reported by Majchrzak, Fabian, and Elmadfa (2006). This ester comprised 45.8% of vitamin A in the liver of pigs from Montanera and the 44.1% for Cebo, being higher for the first group ($p < 0.05$). All-trans-retinol content represented around 15% of the total content of Vitamin A, corresponding the highest value ($p < 0.01$) to Iberian pigs fed on *Montanera* (17.3% vs 12.9%). Moreover, retinyl linoleate was about 6% of total esters and was higher ($p < 0.05$) in M than in C. Finally, neither retinyl oleate concentration nor retinyl stearate showed significant differences between both groups of pigs.

Table 3
Mean values (mg/g), standard deviation and ANOVA for liver retinol and retinyl esters in the two groups of animals included in the study.

Variable	Montanera	Cebo
All-trans-retinol	0.37 ± 0.28	0.12 ± 0.03*
Retinyl linoleate	0.15 ± 0.11	0.05 ± 0.03*
Retinyl oleate	0.45 ± 0.32	0.25 ± 0.10
Retinyl palmitate	0.98 ± 0.65	0.41 ± 0.17*
Retinyl stearate	0.19 ± 0.13	0.10 ± 0.04

Sig.: significant differences. *$p < 0.05$, n.s.: not significant.

On the other hand, Table 4 shows all-trans-retinol levels in the plasma and fat samples as well as the colour parameters in perirenal fat. No significant differences in the concentration of plasma and liver retinol levels were observed. Thus, it could be concluded that retinol in plasma and liver are not useful to differentiate Iberian pigs according to the feeding system. However, significant differences ($p < 0.01$) were observed in the colour parameter L* in the perirenal fat as a function of the animal feeding system, whereas the other variables (a*, b*, C_{ab}* and h_{ab}) did not show differences in this sense. These results are in agreement with those of Carrapiso and García (2005) who reported that the rearing system had a significant influence in the colour parameters of subcutaneous fat of Iberian hams, being L* and C_{ab}* the most affected variables. So the utility of lightness (L*) to discriminate between *Monanera* and *Cebo* feeding-system in Iberian pigs should be further explored.

As explained in the Material & methods section, the reflectance spectra in the visible region (approx. between 360 and 740 nm, considering 10 nm increments) were also acquired and recorded in order to obtain translated reflectance values (TR_i) and the absolute value of the integral of the translated spectrum (AVI). The average translated reflectance spectra (visible region) of the perirenal fat of the two groups of pigs studied are shown in Fig. 2. Previous studies (Ripoll et al., 2008) in lambs with two different feeding systems (grazing vs. concentrate) showed that the translated spectra between 450 and 510 nm (region where carotenoids absorb light) of the perirenal fat were different. Therefore the authors proposed the *translated fat spectrum* as a feeding traceability index in lambs. However, in the present study the TRi (from 450 to 510 nm) of the two groups (Fig. 2) were not different, probably due to the absence of carotenoids in the fat of the animals studied. On the other hand, other studies (Ripoll et al., 2008; Zawadzki et al., 2013) showed higher values for AVI in grazing lambs than in those fed on concentrate, and proposed AVI also as a traceability index in lambs. In the present study no differences were observed between the groups M (41.57 ± 4.15) and C (43.05 ± 7.82) for AVI. Thus, in contrast with ruminant species which absorb carotenoids, neither the reflectance spectra nor the AVI value seemed to be useful to differentiate pigs according to the diet.

In order to assess the utility of the studied parameters to discriminate between feeding systems, a discriminant analysis was carried out considering on one hand the liver retinoids levels and on the other hand the colour parameters in the perirenal fat (Table 5). Retinyl esters as well as all-trans-retinol content in liver were included in the model,

Table 4
Mean values, standard deviation and ANOVAfor the all-trans-retinol levels in plasma (μg retinol/mL plasma), retinol levels in fat (μg retinol/g fat) and colour parameters (L*, a*, b*, C_{ab}* and h_{ab}) measured in the fat of the two groups of animals.

Variable	Montanera	Cebo
Retinol (plasma)	1.07 ± 0.69	0.68 ± 0.38
Retinol (fat)	4.14 ± 1.47	3.69 ± 0.77
L*	57.55 ± 10.66	69.55 ± 13.15**
a*	2.36 ± 2.34	0.94 ± 2.59
b*	9.96 ± 3.52	8.36 ± 2.38
C*	10.48 ± 3.76	8.69 ± 2.78
h_{ab}	81.51 ± 14.56	87.13 ± 12.98

Sig.: significant differences. **$p < 0.01$.

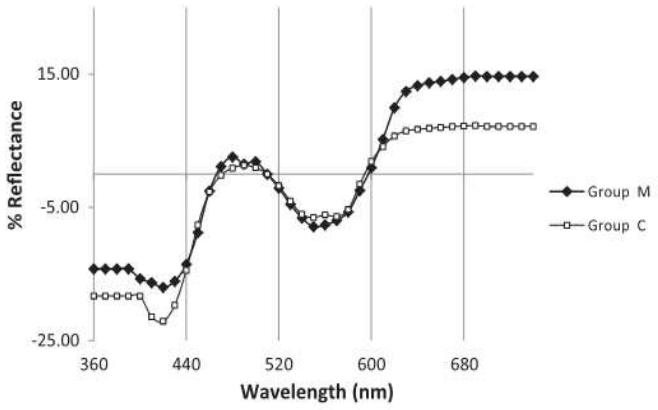

Fig. 2. Averaged translated reflectance spectra (AVI) between 360 and 740 nm of perirenal fat of the two groups of animal studied: group M (Iberian pigs fed on acorns and grass) and group C (Iberian pigs fed on concentrate).

which allowed a correct classification of 92.9% of the animals. More specifically all the pigs reared in an extensive system (group M) were correctly classified (100% of the cases) according to their diet. Furthermore, most of the animals reared indoors were, also, correctly classified (83.3%). These results seem to indicate that the retinyl esters and all-trans-retinol concentration in the liver of Iberian pigs could be a useful parameter to discriminate these animals according to their feeding system. This is in agreement with a previous study (Majchrzak et al., 2006) that concluded that the variations of liver vitamin A concentrations in pigs were related to differences in the vitamin A in the supplied feed. On the other hand, when the colour parameters were considered only L^* and h_{ab} were included in the model. This model allowed classifying correctly up to 78.6% of the animals according to their diet. More specifically, 87.5% of the Iberian pigs on *Montanera* and 66.7% of those fed on concentrate were accurately classified. Thus, it could be said that colour parameters (L^* and h_{ab}) are able to differentiate Iberian pigs according to their diet, so they could be used for traceability purposes.

Finally, in order to reduce the dimensionality of the data set, a PCA was conducted and linear combinations of the original variables were generated. Two principal components or PCs were selected and ranked by decreasing percentages of the total variance explained. The corresponding eigenvector or loadings for each variable included in the model are shown in Table 6. The total variance explained by these 2 PCs was 73.5%. Besides, these PCs could be interpreted by the correlations between them and the original variables. In this sense, PC1 explained 37.5% of the total variance and was indicative of the fat colour since it was positively correlated with the colour variables a^*, C_{ab}^* and b^*. PC2 explained 36.0% of the total variance and had a strong and positive correlation with the variables retinyl palmitate, retinyl stearate, retinyl linoleate, retinyl oleate and all-trans-retinol in liver. The retinol levels in plasma and fat did not correlated with any of the PCs selected. Furthermore, Fig. 3 summarizes the location of each group of animals (those fed on *Montanera* system and those fed on *Cebo*) in the multivariate space formed by the two principal component score vectors.

Table 5
Classification matrix after the discriminant analysis based on liver retinol and retinylesters levels and fat colour variables: assigned (columns) against real data (rows).

Predicted group (%)			
	Group	Montanera	Cebo
Liver retinoids[a]	*Montanera*	100.0	0.0
	Cebo	16.7	83.3
Fat colour[b]	*Montanera*	87.5	12.5
	Cebo	33.3	66.7

[a] 92.9% of cases correctly classified considering the concentration of all-trans-retinol and four retinyl esters in liver samples.
[b] 78.6% of cases correctly classified considering L^* and h_{ab} values in fat.

Table 6
Varimax rotated factor loadings for two groups of animals (Iberian pigs fed on acorns and grass (*Montanera*) and Iberian pigs fed on concentrate (*Cebo*).

Variable	PC1	PC2
h_{ab}(fat)	−.968	−.154
a^* (fat)	.960	.157
C^* (fat)	.948	.218
b^* (fat)	.927	.230
L^* (fat)	−.900	−.199
Retinyl linoleate (liver)	.305	.945
Retinyl palmitate (liver)	.303	.944
Retinyl stearate (liver)	.299	.942
Retinyl oleate (liver)	.301	.926
All-*t*-retinol (liver)	.288	.740

4. Conclusions

Retinol levels in plasma were similar in *Montanera* and *Cebo* animals. On the contrary, retinoids in liver showed significant differences and allowed a good discrimination between groups (92.9% of the animal accurately classified). On the other hand, according to our results, some CIELAB colour parameters (L^* and h_{ab}) of the perirenal fat discriminated correctly 78.6% of the animals according to their diet. What is more, L^* values for the Iberian pigs reared on *Montanera* feeding system were significantly different ($P < 0.01$) from those fed with a diet based on concentrate. Therefore, the colour parameters in perirenal fat could also be useful to differentiate Iberian pigs according to their diet.

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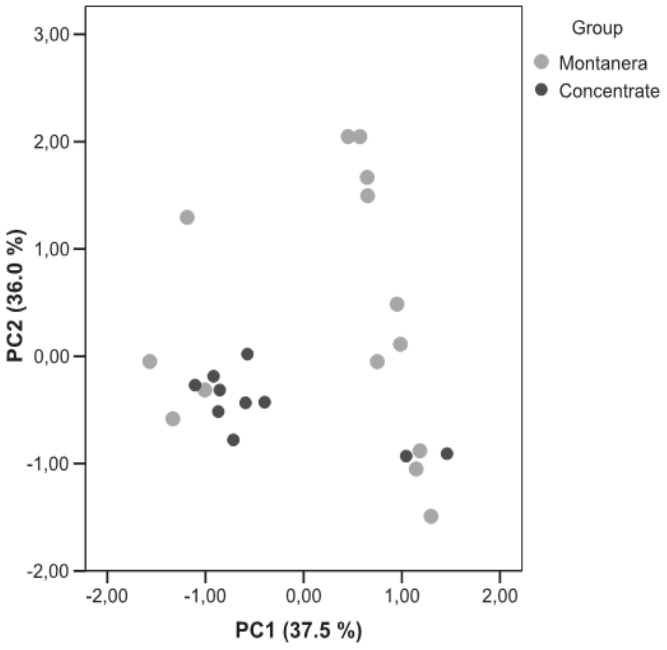

Fig. 3. Principal component analysis of two groups of animals (Iberian pigs fed on acorns and grass (*Montanera*) and Iberian pigs fed on concentrate (*Cebo*)) scores for the variables defining PC1 (a^*, C_{ab}^* and b^* in fat) and PC2 (retinyl palmitate, retinyl stearate, retinyl linoleate, retinyl oleate and all-trans-retinol in liver).

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Chapter 4 *Carotenoids and fat-soluble vitamins in horse tissues. A comparison with cattle.*

ANTECEDENTES

Según Schweigert (1998) y en base a la acumulación de carotenoides en el tejido adiposo, los mamíferos pueden dividirse en dos grupos: "white-fat", que no absorben carotenoides como el cerdo, la cabra, la oveja y los roedores; y los animales "yellow-fat", que sí absorben carotenoides el vacuno, los caballos y las aves están comprendidos en este grupo. Sin embargo, las razones de las diferencias entre especies en el metabolismo de los carotenoides no se conocen bien todavía.

Muchos estudios (Calderón et al., 2007; Majchrzak et al, 2006;.. Martin et al, 2004) han evaluado la acumulación de carotenoides y vitaminas liposolubles (retinol y α-tocoferol) en los fluidos y tejidos de vacuno (plasma, leche, grasa, hígado, etc), así como su implicación en el punto de vista de los consumidores respecto a los productos de vacuno. Sin embargo, existen pocos estudios con este objetivo en caballos (Schweigert y Gottwald, 1999), a pesar de la ventaja que podría suponer la mejora de la aceptabilidad de los productos equinos por parte de los consumidores basándose en la presencia de compuestos que promueven la salud (como los carotenoides y las vitaminas liposolubles), cubriendo la necesidad actual de aumentar el consumo de productos del caballo (Lorenzo et al., 2013). Además, la percepción de los colores juega un papel importante en la evaluación de los consumidores en la calidad de los alimentos (O'Sullivan et al., 2003), pero existe poca información disponible acerca de los productos equinos en este sentido.

OBJETIVOS

Los objetivos de este estudio fueron:

1. Evaluar la acumulación de carotenoides, retinol y α-tocoferol en fluidos biológicos y tejidos en caballo en comparación con el vacuno.
2. Estudiar las diferencias en el color de la grasa entre las dos especies en relación a su composición en carotenoides.

DISEÑO EXPERIMENTAL

Cuatro grupos de animales, de 15 animales cada uno, se utilizaron en este estudio. El Grupo 1 (G1) incluía 15 yeguas lactantes criadas en un sistema extensivo basado en pastos. El Grupo 2 (G2) estaba compuesto por 15 vacas lactantes criadas, también, en un sistema extensivo. Los animales del Grupo 3 (G3) eran potros machos con una dieta basada en alimentos concentrados. Por último, el Grupo 4 (G4) estaba formado por terneros machos con una dieta basada, también, en alimentos concentrados. Se recogieron muestras representativas de todas las dietas. También se cogió sangre de todos los animales, leche de las yeguas (G1) y las vacas (G2), así como muestras de hígado y grasa perirenal de los potros (G3) y los terneros (G4) durante el sacrificio.

Se midió el color de la grasa perirrenal (L *, a *, b *, C * ab y hab) de acuerdo al sistema CIELab (CIE, 1976). Además, también se adquirieron los espectros de reflectancia en la región visible y se transformaron (TR) para calcular el Valor Absoluto de la Integral (AVI, por sus siglas en inglés) de acuerdo a trabajos anteriores (Prache y Theriez, 1999; Priolo et al., 2002; Ripoll et. al, 2008).

Se hicieron extracciones de carotenoides, retinol y α-tocoferoles en todas las muestras. Para los alimentos se siguió la metodología descrita por Pickworth et al. (2012). Las muestras de plasma se extrajeron siguiendo la metodología descrita por Lyan et al. (2001).

Para las extracciones de leche se consideraron los métodos descritos por Hulshof et al. (2006) y Nozière et al. (2006b). En el caso de los hígados, se siguió la metodología descrita por Woodall et al. (1996). Finalmente, las extracciones de grasa perirrenal se llevaron a cabo de acuerdo con Dunne et al. (2006). El contenido en carotenoides, retinoides y α-tocoferol se analizó por HPLC para todas las muestras. Los ésteres de retinol en el hígado se identificaron mediante análisis por HPLC-MS.

RESULTADOS

Las dietas de los animales con el mismo sistema de producción, es decir extensivo (G1 y G2) o intensivo (G3 y G4), mostraron perfiles de carotenoides y α-tocoferol similares, mientras que no se detectó retinol en ninguna de las dietas estudiadas.

Aparecieron diferencias significativas en los niveles de carotenoides en plasma entre yeguas y vacas, siendo la concentración de (13*Z*) -β-caroteno y (todo-*E*)-β-caroteno más alta ($P < 0{,}001$) para las vacas que para las yeguas. Además, se detectó luteína en el plasma de las vacas, pero no en el de las yeguas. Por lo tanto, a pesar de que no había diferencias en las dietas, había diferencias en los niveles circulantes de carotenoides entre bovinos y equinos. Por otro lado, tanto el retinol como el α-tocoferol aparecieron en el plasma de ambas especies. Aunque no se observaron diferencias significativas en los niveles de retinol entre ambas especies, hecho que apoya la hipótesis de la regulación metabólica en la absorción de la vitamina A en ambas especies

Con respecto a la leche, el único carotenoide detectado en ambas especies fue β-caroteno, siendo su concentración significativamente mayor ($P < 0{,}05$) en las vacas que en las yeguas. Estos niveles bajos, a pesar de la elevada presencia de β-caroteno en los alimentos y en el plasma de vacas y yeguas, nos sugieren una transformación similar y limitada de β-caroteno en retinol en ambas especies. Finalmente, el α-tocoferol estaba

presente en la leche de vaca pero no en la de yegua, aunque son necesarios más estudios para establecer el metabolismo de α-tocoferol en ambas especies con el fin de entender las diferencias entre las dos.

Tanto en el plasma de los potros como en el de los terneros se detectaron β-caroteno y retinol pero no α-tocoferol, probablemente debido a la ausencia de esta vitamina en el alimento concentrado de ambas especies. Con respecto a los niveles de β-caroteno, el plasma de los terneros (G4) mostró los niveles más altos ($P < 0,05$) que el de los potros (G3), probablemente debido a la diferencia mínima en el contenido en isómeros de β-caroteno en los piensos de ambas especies . Sin embargo, no se observaron diferencias significativas en los niveles plasmáticos de retinol entre potros (G3) y terneros (G4). Por lo tanto, se podría afirmar que la concentración de carotenoides en plasma de ambas especies depende de la dieta, mientras que los niveles de retinol son regulados de manera eficiente por ambas especies, independientemente de la dieta.

No se encontraron carotenoides en la grasa ni de los potros (G3) ni de los terneros (G4), pero sí se detectó retinol. Este hecho puede ser debido a la mínima cantidad de carotenoides en las dietas de estos animales que podría resultar en cantidades insignificantes de estos compuestos en su grasa. Además, no se detectó α-tocoferol en la grasa de estos animales probablemente debido a su ausencia en la dieta. Por otro lado, todos los parámetros de color medidos en la grasa perirrenal mostraron diferencias significativas ($P < 0.01$, $P < 0,001$) entre ambas especies. El porcentaje de reflectancia de los espectros en la región entre 450 y 510 nm y los valores de AVI fueron mayores ($P < 0.01$) para los potros que para los terneros, lo que nos lleva a pensar que en los potros acumulan más carotenoides en la grasa perirrenal que los terneros, aunque se necesitan más estudios para confirmarlo.

Por otra parte, el contenido en vitamina A en el hígado de ternero fue menor (*P <0,001*) que en el de potro. La vitamina A estaba presente como (todo-*t*)-retinol y los ésteres de retinol linolenato de retinol, linoleato de retinol, oleato de retinol, palmitato de retinol y estearato de retinol. El palmitato de retinol fue la principal forma de vitamina A para ambas especies. Por otra parte, el perfil de ésteres de retinol fue diferente para ambas especies.

CONCLUSIONES

De acuerdo con los resultados obtenidos en este estudio, las principales conclusiones son:

1. Existen diferencias específicas por especie en la acumulación de carotenoides, retinoides y α-tocoferol entre el ganado equino y bovino
2. Los potros acumularon mejor retinoides en el hígado que los terneros.
3. El hígado de potro puede ser considerada como una buena fuente de vitamina A, lo cual debe ser tenido en cuenta desde el punto de vista nutricional para los seres humanos.

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Carotenoids and fat-soluble vitamins in horse tissues. A comparison with cattle

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Abstract:	Carotenoids are important in human health because of their provitamin A function among other biological actions. Their implication on consumer point of view of cattle products have been widely studied, but few information is available for horse products. The aim of this study was to study the accumulation of carotenoids, retinoids and tocopherol by HPLC and HPLC-MS analysis, in different horse tissues (plasma, milk, adipose tissue and liver) and compare it to that of cattle. Fat colour was also studied. Four groups of animals were studied (15 animals within each group): lactating mares (709.82 ± 23.09 kg) and cows (576.93 ± 31.94 kg) reared outdoors, and foals (556.8 ± 25.9 kg, 14 months old) and calves (474.7 ± 36.2 kg, 14 months old) reared indoors were considered. Both mares and foals were from Hispano-Breton breed, while both cows and calves belonged to commercial crossbred Limousine-Retinta Differences in plasma and milk carotenoids ($P<0.05$, $P<0.001$) were found between mares and cows. Similar accumulation of vitamin A in plasma and fat was detected for foals and calves ($P>0.05$). Both species showed a different accumulation of retinoids in liver, being the foal which better accumulate them ($P<0.01$, $P<0.001$). These results indicated that there are species-specific differences in the accumulation of carotenoids, retinol and tocopherol but more studies are needed to establish the mechanism of these differences.

Carotenoids and fat-soluble vitamins in horse tissues. A comparison with cattle.

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Running head: Carotenoids and vitamins in horse tissues.

Abstract

Carotenoids are important in human health because of their provitamin A function among other biological actions. Their implication on consumer point of view of cattle products have been widely studied, but few information is available for horse products. The aim of this study was to study the accumulation of carotenoids, retinoids and tocopherol by HPLC and HPLC-MS analysis, in different horse tissues (plasma, milk, adipose tissue and liver) and compare it to that of cattle. Fat colour was also studied. Four groups of animals were studied (15 animals within each group): lactating mares (709.82 ± 23.09 kg) and cows (576.93 ± 31.94 kg) reared outdoors, and foals (556.8 ± 25.9 kg, 14 months old) and calves (474.7 ± 36.2 kg, 14 months old) reared indoors were considered. Both mares and foals were from *Hispano-Breton* breed, while both cows and calves belonged to commercial crossbred *Limousine-Retinta* Differences in plasma and milk carotenoids ($P<0.05$, $P<0.001$) were found between mares and cows. Similar accumulation of vitamin A in plasma and fat was detected for foals and calves ($P>0.05$). Both species showed a different accumulation of retinoids in liver, being the foal which better accumulate them ($P<0.01$, $P<0.001$). These results indicated that there are species-specific differences in the accumulation of carotenoids, retinol and tocopherol but more studies are needed to establish the mechanism of these differences.

Keywords: Horse; Bovine; Carotenoids; Retinoids; Liver

Implications

The overproduction of horses in Europe is claiming to promote the consumption of equine products (Alvarez *et al.*, 2013, Saastamoinen, 2013). Thus, it will be interesting to value equine alternative productions because of the increase of animals slaughtered, which implies an increment of equine products in the market. The study of health-promoting compounds in equine products like carotenoids and fat-soluble vitamins (retinol and tocopherol) would help to improve this consumption. In this paper, the accumulation of these compounds in different horse tissues (plasma, milk, fat and liver) have been explored in comparison to that of cattle, widely studied by previous authors. Absorption and accumulation of carotenoids, retinol and tocopherol were observed in horse fluids and tissues.

Introduction

Carotenoids are important in human health and nutrition because of their provitamin A function and other possible biological actions. In this sense, they have been associated with a lower risk of developing certain types of cancer, cardiovascular diseases (CVD), cataracts, etc. These compounds are lipophilic pigments that animals cannot synthesize them *di novo* but they are able to metabolize them, such that those present in their plasma or tissues come from the diets (Nozière *et al.*, 2006a). In addition, carotenoids with at least an unsubstituted β-ring in their structure (such as β-carotene, α-carotene and β-cryptoxanthin) are precursors of vitamin A, i.e. retinol, through cleavage. Retinol is involved in several functions, such as vision, growth and bone development, reproduction and integrity of mucosal and epithelial surfaces (Ortega *et al.*, 2005). Schweigert (1998) established that based on the accumulation of carotenoids in adipose tissue, mammals can be divided into two groups: the

57 "white-fat" and the "yellow-fat" animals. The first group is comprised by species
58 that do not absorb carotenoids at all or at least only at very low levels such as
59 pig, goat, sheep and rodents. Animals in second group are those that do absorb
60 carotenoids such as cattle, horses and birds. However, reasons for the
61 characteristic species-specific differences in carotenoid metabolism are not yet
62 well understood.

63 On the other hand, the role of fat-soluble vitamins (such as retinol and
64 tocopherol) in the nutritional and sensory properties of foods have been recently
65 pointed out (Sauvant *et al.*, 2011). Many studies (Martin *et al.*, 2004; Majchrzak
66 *et al.*, 2006; Calderón *et al.*, 2007) have been carried out about the presence
67 and accumulation of carotenoids and fat-soluble vitamins in cattle fluids and
68 tissues (plasma, milk, fat, liver, etc) as well as their implication on consumer
69 point of view of the cattle products. However, few studies have been developed
70 (Schweigert & Gottwald, 1999) with this aim in horses. Therefore, it would be
71 interesting to study the accumulation of both carotenoids and fat-soluble
72 vitamins in horse tissues since, nowadays (Lorenzo *et al.*, 2014) efforts in
73 extending the knowledge on equine products by improving consumer
74 awareness of the high value of these products are being carried out, since in
75 countries like Spain their production is growing but the consumption is limited
76 (Lorenzo *et al.*, 2013). So, the study of health-promoting compounds like
77 carotenoids could help increase the acceptability of horse products by the
78 consumer in order to improve their consumption in countries like Spain, Italy,
79 France and Belgium where it remains low (Lorenzo *et al.*, 2014).In addition, it
80 has been stated that the colour perception of food quality play a major role in
81 consumer evaluation (O'Sullivan *et al.*, 2003). In this way, consumer needs first

to be entirely satisfied with the sensory properties of products, before other quality dimensions become relevant. Studies of colour fat in different species, such as cattle, have been carried out (Dunne *et al.*, 2009) but limited information is available about horses in this sense.

In this regard, the aim of this report was to study the accumulation of carotenoids and fat-soluble vitamins (retinol and tocopherol) in different horse tissues compared to that of cattle at two levels, females fed on pasture and young males fed indoors and tied. Differences in fat colour were also assessed with the same aim.

Material and methods

Animals

For this study, four groups of animals were used. Group 1 (G1) included 15 lactating mares from *Hispano-Breton* breed, whose rearing is oriented to meat production (www.feagas.com). These animals had an average weight of 709.82 ± 23.09 kg and they were reared in an extensive feeding system. Group 2 (G2) was composed by 15 lactating cows in an extensive production system, with an average weight of 576.93 ± 31.94 kg. All animals belonged to a commercial crossbred *Limousine-Retinta* mainly intended for meat production (www.feagas.com). Group 3 (G3) was composed by 15 male foals from *Hispano-Breton* breed. The foals were weaned at 6 months old and fattened in an intensive system until the moment of the slaughter (around 14 months old) when the animals had an average weight of 556.8 ± 25.9 kg. Finally, group 4 (G4) consisted on 15 male calves from a commercial crossbred *Limousine-Retinta*. These animals were weaned at 6 months old and fattened in

106 confinement until they were 14 months old, when they were slaughtered with an
107 average weight of 474.7 ± 36.2 kg.

108 *Diets and sampling* Mares from G1 were reared in a continuous grazing
109 system in an area of dehesa silvopastoral system in Central Spain Mountains,
110 where pasture has been characterized by previous authors (San Miguel *et al.*,
111 2009) as xero-mesophytic acid soil. What is more, Martínez *et al.* (2014)
112 reported 1159 Kg DM/ha as biomass production in an area similar to that where
113 our animals were reared, with 12.52% Crude Protein and 7.3% legumes. These
114 pastures allow stocking between 0.5-1.0 Livestock Unit/ha. The paddock where
115 the mares were reared was mainly composed, accordingly to Muslera and
116 Ratera (1984), by plants from *Poaceae* family, like *Agrostis spp.*, *Bromus spp.*,
117 *Festuca spp.* Besides species from *Fabaceae* family were present like *Trifolium*
118 *spp.*, *Medicago spp.* and *Lotus spp.* In addition, species from *Asteraceae* family
119 like *Carduus spp.* were present. Furthermore, cows from G2 were reared based
120 on continuous grazing system in a farm in Southwestern Spain in a dehesa
121 landscape. This area is characterized by a Mediterranean climate with mostly
122 acid soils on siliceous sedimentary materials (Alejano *et al.*, 2012). Besides with
123 1440 Kg DM/ha as average production (10.3% Crude Protein and 8.5%
124 Legumes). which led a similar central-Spain dehesa stocking between 0.5-1.0
125 Livestock Unit/ha. In the case of the paddock where the animals were reared,
126 the pasture was mainly composed accordingly to Madejón *et al.* (2009), of
127 plants from *Poaceae* family, like *Agrostis spp.*, *Vulpia app.*, *Poa spp.*, *Bromus*
128 *spp.* and *Lollium spp.* Besides, species from *Fabaceae* family were present, like
129 *Trifolium spp.*, *Ornithopus spp.*, *Medicago spp.* and *Lotus spp.* The amount of
130 pasture ingested by each animal, both mares and cows, was not controlled

since they presented a good body condition due to a proper stocking adapted to the pasture production.

According to Olea and Paredes (1997) most of pasture production in these areas (70%) occurs in spring, so representative samples of the different pastures from G1 and G2 were collected specifically in mid-May, ten days before the sampling of the animal tissues since a low persistence of carotenoids in blood of animals have been reported previously (Prache *et al.*, 2003b; Serrano *et al.*, 2007). The sampling areas were randomly selected at the prairie where the animals were reared. The pasture was sampled by triplicate from the ground using six definite quadrates (0.5 m by 0.5 m) randomly established. Samples were pooled in order to analyze them overall because of the reported (Nozière *et al.*, 2006a) variability in carotenoid content between botanical species. On the other hand, concentrate fed to foals (G3) was composed (Piensos Binaga S.A., Huesca, Spain) of barley, soybean, carob flour, maize gluten, bran leaf, beet pulp, molasses, palm oil, calcium carbonate, sodium chloride, phosphate dicalcium. The analytical composition of the concentrate was as follows: crude protein (14.3%), Mg (0.22%), fat (3.4%), Ca (0.89%), crude fiber (11.0%), ash (6.1%) and P (0.50%). Finally, the diet (Piensos Inalsa S.A., Ciudad Real, Spain) of the calves (G4) was composed by maize, barley, soybean, citric pulp, wheat, calcium carbonate, sodium chloride, fatty acids, calcium carbonate, palm oil. The analytical composition was: crude protein (15.5 %), fat (4.7 %), crude fiber (4.5 %), ash (5.6 %) and sodium (0.33 %).

After collecting, all the feedstuff samples were transported to the laboratory, lyophilized and stored at -80°C until extraction. Samples were analyzed in triplicate according to a methodology described by Pickworth *et al.* (2012) with

156 some modifications. 1 g. of each sample was lyophilized and the extractions
157 were carried out on the extract with 3 mL of hexane/ethanol (1:1, v/v) as
158 extractant. The extraction was repeated twice and the organic phases obtained
159 were pooled and saponified with 15% ethanolic potassium hydroxide (w/v)
160 overnight at room temperature in the dark and under nitrogen atmosphere.
161 Finally, the organic phase was washed several times with water. After that, the
162 organic phase was collected and dried using a concentrator (Concentrator plus,
163 Eppendorf Research®, Madrid, Spain). The dry residue was dissolved in 100 µl
164 of ethyl acetate for HPLC analysis. Table 1 summarizes the content on
165 carotenoids and α-tocopherol of the diets of the animals. The main carotenoids
166 in pasture (G1 and G2) were lutein, β-carotene and its *Z* isomers, while lutein
167 and β-carotene were detected in concentrate samples from both G3 and G4, as
168 well as zeaxanthin and (9Z)-β-carotene in concentrate from G4. Retinol was not
169 detected neither in pasture nor in concentrate. However, α-tocopherol was
170 present in pasture but not in concentrate. Considering the data in Table 1 it can
171 be stated the diets of the animals in this study with the same rearing system, i.e.
172 extensive or intensive, showed a similar carotenoid and fat-soluble vitamins
173 profile

174 *Tissues of mares and cows*

175 Thirty milliliters (mL) of milk were collected from mares and cows (G1 and
176 G2). For this, the animals were placed in a cattle crush in order to make the
177 handing easier. The milk was collected aseptically into sterile vials covered with
178 aluminum folio and immediately stored at -80°C until laboratory analysis.
179 Besides, ten mL of blood were taken, using Li-Heparin as anticoagulant, from
180 the jugular vein and the caudal vein of the mares and cows, respectively. Blood

181 samples were transported to the laboratory at 4°C to centrifuge them (1500 g,
182 10 min, 4 ° C) and collect the plasma which was stored at -80°C until analysis.
183 The extraction of carotenoids and fat-soluble vitamins from milk was carried
184 out applying approved methodologies by Hulshof *et al.* (2006) and Nozière *et al.*
185 (2006b) with some modifications. 1 mL of milk was mixed with 0.5 mL of distilled
186 water, 1 mL of ethanol and 1 mL of hexane. Samples were then centrifuged
187 (8000 g, 10 min, 4°C) and the upper organic phase collected. The extraction
188 was repeated until colour exhaustion. The organic phases were pooled and 2
189 mL of ethanolic sodium hydroxide 15% (w/v) were added. The saponification
190 reaction was maintained overnight at room temperature in the dark. Finally, the
191 organic phase was washed several times with water, collected and dried using
192 a concentrator (Concentrator plus, Eppendorf Research®, Madrid, Spain), The
193 dry extract was dissolved in 35 µl of ethyl acetate for HPLC analysis.
194 For plasma samples, the methodology described by Lyan *et al.* (2001) was
195 used with some modifications. 2 mL of plasma were deproteinized by adding 2
196 mL of ethanol. Carotenoids, retinol and tocopherol were extracted twice with 2
197 mL of hexane with centrifugation (8000 g, 10 min, 4°C); the extracts were
198 pooled and evaporated to dryness with a concentrator (Concentrator plus,
199 Eppendorf Research®, Madrid, Spain). The dried extracts were dissolved in 100
200 µl of ethyl acetate for their injection on the HPLC system.

201 *Tissues of foals and calves*

202 Foals and calves (G3 and G4) were slaughtered in spring according to the
203 European Regulation (CE No 1099/2009 of 24 September 2009). The animals
204 arrived at the abattoir the night before the slaughter and had access to water
205 until 30 minutes before slaughtering. They were transported to the abattoir by

206 truck in agreement with EU Regulation (CE No 1/2005 of 22 December 2004).
207 The slaughterhouse was located 9 km (G3) and 12 km (G4) from the farms
208 where the animals were reared. Ten mL of blood were taken from each animal
209 at the moment of the slaughter using Li-Heparin as anticoagulant. In addition,
210 three grams (g.) of liver and five of fat from the perirenal area of the carcass of
211 each animal were also collected just after the slaughter. This location was
212 selected based on the hypothesis of Priolo *et al.* (2002) who affirmed a greater
213 accumulation of carotenoid pigments in perirenal compared to caudal fat in
214 lambs. One hour after the slaughter, the instrumental color measurements of
215 the perirenal fat samples were carried out. The samples were transported to the
216 laboratory at 4°C, the blood was centrifuged (1500 g, 10 min, 4°C) and the
217 plasma was collected. All samples were stored at -80°C until further analysis.
218 Prior to the analysis, all the samples were thawed overnight and in the dark in a
219 fridge at 4°C.
220 The perirenal fat CIELab (CIE, 1976) colour parameters (L^*, a^*, b^*, C^*_{ab} and
221 h_{ab}) were measured on a CM-700d spectrocolorimeter (Konica Minolta
222 Holdings, Inc, Osaka, Japan) considering the D_{65} Illuminant, the 10º Observer
223 and zero and white calibration. The reflectance spectra on the visible region
224 (approx. between 360 and 740 nm, considering 10 nm increments) were also
225 acquired and recorded in order to obtain translated reflectance values (TR_i) and
226 the absolute value of the integral (AVI) of the translated spectra. In previous
227 studies (Priolo *et al.*, 2002) the reflectance spectra between 510 and 450 nm
228 were translated to make the reflectance value at 510 nm equal to zero (TR).
229 The TR_i were calculated from the reflectance values (R_i) as follows: $TR_i = R_i -$

230 R_{510} with i = 360, 370, 380...740; whereas AVI of the translated spectra were
231 calculated according to the following formula:
232 $$AVI = [(TR_{450}/2) + TR_{460} + TR_{470} + TR_{480} + TR_{490} + TR_{500} + TR_{510}/2] \times 10$$
233 Carotenoids and fat-soluble extractions in plasma was carried out following the
234 same methodology explained above for plasma of mares and cows. For liver
235 samples, the methodology described by Woodall *et al.* (1996) was followed with
236 some modifications. 0.1 g of liver was lyophilized and the dried samples were
237 homogenized in 0.5 mL of saline solution 85% (w/v). The compounds were
238 extracted from the samples using 1 mL of dichloromethane by vortexing for 1
239 min, centrifuging (8000 g, 10 min at 4°C) and collecting the lower layer.
240 Samples were extracted two times, pooled and evaporated. After that, the
241 extracts were reconstituted with 35 µl of ethyl acetate for the injection on the
242 HPLC. Finally, fat extraction was carried out following the methodology
243 described by Dunne *et al.* (2006) with some modifications. 500 mg of perirenal
244 fat were mixed with 1 mL of 3,5-di-ter-4-butylhydroxytoluene (BHT) solution
245 (12% w/v) in order to suppress the oxidation of the analytes. 5 mL of ethanolic
246 sodium hydroxide solution (30% w/v) and 5 mL of ethanol were also added. The
247 saponification reaction was carried out overnight at room temperature and in the
248 dark. Water was finally added to stop it and the analytes were extracted with 10
249 mL of a mixture ether/hexane (2:1 v/v). The samples were then centrifuged
250 (8000 g, 10 min, 4°C) and the upper organic phase collected. The extraction
251 was repeated twice. The organic phases were pooled, washed several times
252 with water, collected and dried. The residue was dissolved in 1 mL of ethyl
253 acetate and filtered for HPLC analysis.
254 *High Performance Liquid Chromatography (HPLC)*

255 The analyses were performed on an Agilent 1100 system (Agilent, Palo Alto,
256 CA, United States) fitted with a photodiode array detector, a quaternary pump, a
257 column temperature control module set at 20°C and an auto sampler set to
258 draw 20 µl aliquots from the concentrated extracts. An YMC C_{30} column (5 µm,
259 250 x 4.6 mm) (YMC, Wilmington, NC, USA) were used for the analyses. The
260 mobile phase consisted of methanol (MeOH), methyl-ter-butyl ether (MTBE)
261 and water according to the linear gradient: 0 min: 90% MeOH + 5% MTBE + 5%
262 water; 12 min: 95% MeOH + 5% MTBE; 25 min: 89% MeOH + 11% MTBE; 40
263 min: 75% MeOH + 25% MTBE; 50 min: 40% MeOH + 60% MTBE; 56 min: 15%
264 MeOH + 85% MTBE; 62 min: 90% MeOH + 5% MTBE + 5% water. The mobile
265 phase was pumped at 1 mL/min and the chromatograms were monitored at 450
266 nm for carotenoids, at 325 nm for retinoids and at 280 nm for tocopherol. The
267 compounds were identified by comparison of their spectroscopic and
268 chromatographic characteristics with those of standards obtained from natural
269 sources by recommended procedures as described elsewhere (Meléndez-
270 Martínez *et al.*, 2009). The quantification was performed by using calibration
271 curves of standard solutions.

272 *Liquid chromatography-electrospray ionization ion trap/time-of-flight mass*
273 *spectrometry*

274 Identification of retinyl esters was done by HPLC-MS analysis according to
275 Álvarez *et al.* (2014). The liquid chromatography in the HPLC-ESI/TOF-MS
276 system was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific,
277 Waltham, MA, USA). The HPLC conditions were the same as described above.
278 An YMC C_{30} column (5 µm, 250 x 4.6 mm) (YMC, Wilmington, NC, USA) was
279 used. A split post-column of 0.4 mL/min was introduced directly on the mass

spectrometer electrospray ion source. Mass spectrometry was performed using a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with Q-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode using a scan range from m/z 50-1200. Mass spectra were acquired in MS full scan. The instrument control was performed using Bruker Daltonics HyStar 3.2.

Statistical analysis

The SPSS 15.0 software for Windows (SPSS Inc., 2006) was used. The analysis of variance (ANOVA) test was applied to assess the existence of significant differences. The significant differences among groups' means were determined by a Tukey-b post hoc test, with a significance level of $P< 0.05$. Correlations analyses were performed between β-carotene levels in plasma and milk of mares and cows, as well as, between AVI and b* values in fat of foals and calves, using the GLM procedure of SPSS. Discriminant analysis was carried out using a stepwise model considering the specie of the animals as independent variable. The discriminant classification method was leave-one-out-cross-validation. The significance level for a variable to be included in the model was 0.05.

Results and Discussion

Carotenoids and vitamins in the tissues of mares and cows

Plasma. (13*Z*)-β-carotene and all-*E*-β-carotene were present (Table 2) in plasma of both species the concentration being significantly higher ($P<0.001$) in cows than in mares, according to the numerical differences between the diets although these differences were not statistically significant. The absence of

305 significant differences in carotenoids content between both pasture diets (Table
306 1) was expected since Nozière *et al.* (2006a) summarized that differences in
307 carotenoids content among plant species are less important that within species
308 differences due to drying, processing or maturity stage, and both pasture diets,
309 for mares and for cows, were from similar growing areas at the same season.
310 Lutein was also detected in cow plasma, but not in mares. In relation to this,
311 dramatic differences in the bioavailability of carotenoids between different
312 species of animals have been reported and it has been pointed out (Schweigert,
313 1998) that some strictly herbivorous species may absorb carotenoids into the
314 mucosa cells of the small intestine, but may not be capable of transferring the
315 carotenoids into the body's circulation. These observations could explain the
316 low plasma carotenoids levels in mares. It must be underlined that our
317 experimental design did not allow to assess the mechanisms of these
318 differences and those observed are global effects of the experimental
319 comparison between both species. In addition, Yang, *et al.* (1992) stated that
320 although xanthophylls account for the majority of dietary carotenoids, β-
321 carotene is the main circulating carotenoid in bovines. However, the
322 mechanisms that could explain the low apparent transfer of xanthophylls form
323 forages to plasma in bovines have not been clearly described. Furthermore,
324 Calderón *et al.* (2007) reported in cows fed on a mixed ration (hay + silage +
325 alfalfa +concentrate) two isomers of β-carotene in plasma, i.e. all-*E*-β-carotene
326 (78.9%) and (13*Z*)-β-carotene (8.6%), these results are in accordance with our
327 results where β-carotene represented the 88.3% and (13*Z*)-β-carotene the 8.2%
328 of total plasma carotenoids in cow plasma. With respect to Plasma Carotenoid
329 Content (PCC) in mares, β-carotene appeared (Table 2) as the main circulating

carotenoid (90.5%), while (13*Z*)-β-carotene appeared in much lower amounts (9.5%). Few information is available in the bibliography with respect to PCC in mares, and it was of our knowledge the first time that plasma carotenoids levels in cows and mares are compared. Taking into consideration these data it can be concluded that, despite there were not differences in the diets (Table 1), there were differences in circulating carotenoids levels between bovine and equine. However, more studies are needed in order to establish the underlying reasons of these differences.

On the other hand, retinol and α-tocopherol appeared (Table 2) in plasma of both species. No significant differences in retinol levels were observed between both species. Previously Greiwe-Crandell *et al.* (1997) reported that grazing horses derive vitamin A from provitamin-A carotenoids present in the forages but the efficiency of this conversion is relatively poor in these animals. Furthermore, it has been stated (Martin *et al.*, 2004) that the levels of vitamin A in blood of cows was unaffected by the diet because of the animal's metabolic regulation. Considering our data (Table 2) it is tempting to hypothesize that this metabolic regulation also occurs in mares. This hypothesis could be supported by the data of Kuhl *et al.* (2012) who stated that a β-carotene supplementation did not increase plasma vitamin A concentrations neither in mares nor in foals.

Milk. β-carotene was the only carotenoid detected in both milks (Table 2), the concentration being significantly higher ($P<0.05$) in cows than in mares. These findings contrast with those reported by Schweigert & Gottwald (1999) who observed that the concentrations of β-carotene in milk of mares are comparable to those of cow. Greiwe-Crandell *et al.* (1997) hypothesized that the bioavailability of β-carotene may be overestimated because of the presence or

absence of other substances in the diet that may influence efficiency of absorption and metabolism, and there are significant differences in β-carotene utilization within species, which could explain the differences found in the present study. Besides, Schweigert & Gottwald (1999) established that β-carotene levels in milk at all stages of lactation are highly dependent on the level in plasma, however we did not observe significant correlations (*P>0.05*) in this sense neither for mare nor for cows. Calderón *et al.* (2007) stated for cattle that when plasma β-carotene exceeds 5 μg/mL the mechanisms involved in the transfer of β-carotene from plasma to milk are limiting in terms of β-carotene secretion. This indicates that the limitation of β-carotene secretion may not be due to a higher cleavage of this carotene in the mammary gland, but rather to a limited uptake by the mammary gland or limited transport by binding β-lactoglobulin and/or to saturation of milk fat globules. This could help to understand the lower levels of β-carotene in cow's milk with respect to plasma since the total β-carotene plasma levels, i.e. all isomers detected, reported in the present study were very close to the limit proposed by Calderón *et al.* (2007). More studies are needed to clear up the mechanism of carotenoids secretion into milk for both species.

Besides, low levels of retinol were also found in milk of both species (Table 2). Mora *et al.* (2000) demonstrated the low intestinal activity of 15.15'-dioxygenase, enzyme responsible of β-carotene conversion into retinal (that is further converted into retinol) in cattle, which may explain to some extent the presence of β-carotene in the milk of cows. This affirmation agrees with the data obtained in the present study for both cow and mare milk which suggested to us that the transformation of carotene to retinol could be similar and limited in both

380 species. Finally, α-tocopherol was present in cow but not in mare milk. Engel *et*
381 *al.* (2007) found a high depletion (3x) of α-tocopherol levels in mares from
382 colostrum to milk until achieving minimal levels of this vitamin at 4 days of
383 lactation (ng/mL). This could explain to some extent the absence of this vitamin
384 in mare milk. Nevertheless, more studies are necessary to establish the
385 metabolism of α-tocopherol in both cattle and horses before trying to clarify the
386 differences between the two species.

387 *Carotenoids and vitamins in the tissues of foals and calves*

388 *Plasma*. When plasma from foals and calves were analyzed β-carotene and
389 retinol were detected but α-tocopherol was not, probably due to the absence of
390 this vitamin in the concentrate fed to both species (Table 1). With respect to β-
391 carotene levels, the plasma from the calves (0.05 ± 0.04 μg/mL) showed higher
392 ($P<0.05$) levels than that from the foals (0.02 ± 0.01 μg/mL), probably due to
393 minimal difference in β-carotene isomers content in the feedstuffs of both
394 species (Table 1). However, these levels of β-carotene were lower than those
395 reported in previous studies (Mäenpää *et al.*, 1988; Gay *et al.*, 2004; Serrano *et*
396 *al.*, 2007) probably due the diet of the animals since Mäenpää et al. (1988)
397 studied foals fed on pasture, Gay *et al.* (2004) studied suckling foal plasmas
398 and Serrano *et al.* (2007) supplied a mixed diet of grass and concentrate.
399 Moreover, Serrano *et al.* (2007) reported that the persistence of carotenoid in
400 plasma is usually low which could also explain the levels found in this study.
401 With respect to retinol levels in plasma, no statistically significant differences
402 were observed between foals (7.41 ± 2.62 μg/mL) and calves (6.20 ± 1.91
403 μg/mL). These levels are remarkable in comparison with those of β-carotene in
404 the plasma of the same animals. In this sense, Schweigert (1998) confirmed

405 that in mammals an estimation of the dietary carotenoid supplementation can
406 be obtained from plasma levels, since carotenoid levels in blood plasma are
407 clearly affected by the dietary intake and reflect the intake of these compounds
408 in recent weeks, contrary to vitamin A which is homeostatically regulated.
409 Therefore, it could be concluded that plasma carotenoid concentration of both
410 foals and calves depend on the diet, while retinol levels are efficiently regulated
411 for both species, independently of the diet.

412 *Fat*. Retinol was detected in fat samples while carotenoids did not (Table 3).
413 This fact could be due to the minor amount of carotenoids in the diets of these
414 animals (Table 1) which could result in negligible amounts of these compounds
415 in their fat. α-Tocopherol was not detected in the fat of the animals either,
416 probably because of its absence in the diet and the fact that young animals
417 accumulate less amount of pigments. Finally, retinol was detected in the fat of
418 both foals (0.16 ± 0.12 mg/g fat) and calves (0.20 ± 0.10 mg/g fat) but not
419 significant differences were found between species. This may indicate that there
420 are certain similarities in the absorption and metabolism of this vitamin between
421 species.

422 Table 3 summarizes the data relative to the colour parameters measured in
423 fat. All parameters showed significant differences ($P<0.01$, $P<0.001$) despite the
424 animals were slaughtered at the same age and they were reared under very
425 similar conditions, indicating that such differences could be due to some extent
426 to factors related to the species. More specifically, b^* and L^* values were
427 significantly higher ($P<0.001$, $P<0.01$, respectively) in foals than in calves,
428 indicating that the fat from foals was lighter and yellower than that from calves.
429 In this sense, Priolo *et al.* (2002) reported that yellow fat is associated with

carotenoid accumulation, so it could be interesting for foal producers, since the "yellow fat" could be associated to a desirable "natural" and health-promoting-rich diet, which could be exploited to increase the consumption of foal meat. Besides, when the percentage of reflectance spectra in the vision region (i.e. from 360 to 740 nm) of the fat from both species was evaluated significant differences ($P<0.01$) were observed in the region between 450 and 510 nm (i.e. in the region where the carotenoids absorb light) between species. Figure 1 shows the average translated reflectance spectra of the perirenal fat of foals and calves, being to our knowledge the first time that foal fat spectrum is measured and compared with that from calf fat. Prache *et al.* (2003a) demonstrated that perirenal fat carotenoid concentration is negatively related to live weight gain during finishing period, while the spectrocolimetric index of perirenal fat remains high during this finishing period, which could support the hypothesis that foal accumulate more carotenoids in fat than calves, although more studies are needed in order to confirm it. Table 3 also shows the mean value of the absolute value of the integral (AVI) of the translated spectra between 450 and 510 nm (the integral values were always negative and for this reason is presented as absolute value) for both species. It could be observed that this AVI was significantly higher ($P<0.01$), in foals than in calves, supporting, too, the hypothesis that there are differences in the accumulation of pigments in perirenal fat between both species. In addition, positive and significant correlations were found out between AVI and b^* values for both foals ($r = 0.830$, $P<0.01$) and calves ($r = 0.839$, $P<0.01$). Priolo *et al.* (2002) also reported positive correlations between these two parameters in perirenal fat in lambs and they attributed them to the carotenoid concentration in fat, so that

455 higher values of b* and AVI were assigned to higher concentration of theses
456 pigments in fat.

457 *Liver.* Wingerath *et al.* (1997) reported that in mammals, up to 80% of the
458 body's total retinol is present in the liver, mainly located in stellate cells,
459 representing the retinyl esters with various long-chain fatty acids the major
460 storage forms. Table 4 shows the data relative to these compounds found in
461 both foal and calf livers, as well as the retinol equivalents (RE) values
462 calculated for both species. The livers of calves contained lower ($P<0.001$)
463 amounts of vitamin A (2.94 ± 1.94 mg RE/100 g) than those from foals (10.05 ±
464 4.95 mg RE/100 g). These results in calves are in good agreement with those
465 obtained by Majchrzak *et al.* (2006) who reported 2.01 ± 0.55 of mg RE/100 g
466 as the mean value of retinol equivalents in calves, however these authors did
467 not report data for foals. In addition, Alosilla *et al.* (2007) reported that vitamin A
468 availability is limited in ruminants due to losses by ruminal destruction, which is
469 especially high when ruminants are fed high concentrate diets. Therefore, under
470 our trial conditions, it can be deduced that foals accumulate retinoids in liver
471 better than calves, so the foal liver may be considered a good source of vitamin
472 A. In the examined livers, vitamin A was present for both species as retinol,
473 retinyl linolenate, linoleate, oleate, palmitate and stearate. Retinyl palmitate was
474 the predominant form of vitamin A in the liver of both foals (47.6%) and calves
475 (37.1%), which is in agreement with Majchrzak *et al.* (2006). In addition, the
476 profile of retinoids, in percentage, was different between species (Table 4),
477 being to our knowledge, the first time that retinyl linolenate is identified in liver
478 from foal and calf. To sum up, the contribution of retinyl esters with saturated
479 fatty acids, i.e. retinyl palmitate and stearate, accounted for 55.4% and 60.1% of

480 total vitamin A in liver of foal and calf, respectively. While the retinyl esters with
481 polyunsaturated fatty acids, i.e. retinyl linoleate and linolenate, made up 26.3%
482 of total vitamin A in foal liver and the 14.6% in calf liver. Finally, the contribution
483 of retinol and retinyl oleate to the total vitamin A in the liver for foals supposed
484 the 11.8% and 6.5% respectively, and the 7.5% and 17.8%, respectively, for
485 calves (Table 4). The difference in the retinyl esters profile might be due to the
486 bacterial modification of dietary fat in the rumen of the calves, i.e. the free
487 released unsaturated fatty acids are hydrogenated to saturated fatty acids
488 (Majchrzak *et al.*, 2006). Finally, a discriminant analysis was carried out in order
489 to assess which retinoids differentiate better the liver from both species. Retinol,
490 retinyl stearate and retinyl palmitate were considered in the model. This model
491 allowed classifying the 100% of the liver samples according to the species (foal
492 *vs.* calf). All these results seem to indicate that the accumulation of retinol and
493 its esters in liver is different between foals and calves reared under the same
494 conditions. This should be taken into consideration as it is interesting from a
495 nutritional point of view for humans.

496 **Conclusions**

497 Species-specific factors exist in accumulation of carotenoids and fat-soluble
498 vitamins (retinol and tocopherol) between equine and cattle livestock. In this
499 sense, differences in circulating and secretion of carotenoids into the milk were
500 found between mares and cows, thus more studies are needed in order to
501 establish the mechanism of this metabolism in horses. Furthermore, similar
502 accumulation of vitamin A in plasma and fat was detected for foals and calves,
503 which implies a homeostatic regulation of it for both species, independently of
504 the diet. Finally, both species have a different metabolism for retinoids in liver,

505 such that the foals have better ability to accumulate them. The foal liver may be
506 considered a good source of vitamin A, which should be taken into
507 consideration from a nutritional point of view for humans.

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676

677 ## Annex 1: Tables

678 **Table 1.** *Level (µg/g DM) of carotenoids and α-tocopherol identified in the diets of the*
679 *animals: Group 1 (pasture of mares), Group 2 (pasture of cows), Group 3 (concentrate*
680 *of foals), Group 4 (concentrate of calves).*

Diet	Pasture (Group 1)	Pasture (Group 2)	Concentrate (Group 3)	Concentrate (Group 4)	Sig.
Violaxanthin	11.71	14.49	n.d.	n.d.	**n.s.**
Lutein	38.57^{b}	52.93^{b}	0.09^{a}	0.42^{a}	*
Zeaxanthin	4.58^{b}	3.28^{b}	n.d.	0.32^{a}	***
***13(Z)*-β-Carotene**	1.90	3.75	n.d.	n.d.	n.s.
β-Carotene	10.92^{b}	12.35^{b}	0.05^{a}	0.17^{a}	*
***9(Z)*-β-Carotene**	3.18	5.10	n.d.	0.07	**n.s.**
α-Tocopherol	59.99	37.34	n.d.	n.d.	**n.s.**

681 Sig.: significant differences. *$P<0.05$, ***$P<0.001$, n.s.: not significant. Significant
682 differences ($p<0.05$) within a column are indicate by superscripts (a, b, c). n.d.: not
683 detected.

684

685

686 **Table 2.** *Carotenoids, retinol and α-tocopherol (μg/mL) detected in plasma and milk*
687 *from mares (Group 1) and cows (Group 2) bothfed on pasture*

Compound	Sample	Mare	Cow	s.e.m	Sig.
Lutein	*Plasma*	Traces	0.16	0.01	**n.s.**
	Milk	n.d.	n.d.		
***(13Z)*-β-Carotene**	*Plasma*	0.07	0.38	0.05	***
	Milk	n.d.	n.d.		
β-Carotene	*Plasma*	0.67	4.07	0.47	***
	Milk	0.09	0.23	0.03	*
Retinol	*Plasma*	6.58	6.35	0.53	**n.s.**
	Milk	1.04	2.85	0.41	*
α- Tocopherol	*Plasma*	1.58	8.11	0.77	***
	Milk	n.d.	1.66	0.30	

688 s.e.m.: standard error of the means. Sig.: significant differences. *$P<0.05$, ***$P<0.001$,
689 n.s.: not significant, n.d.: not detected

690

691

692 **Table 3. R***etinol (mg/g fat), colour parameters (L*, a*, b*, C^*_{ab}, h_{ab}) and Absolute Value*
693 *of the Integral (AVI) of the translated reflectance spectra measured in the fat of foals*
694 *(Group 3) and calves (Group 4) with a diet based on concentrate.*

Variables	Foal	Calf	s.e.m.	Sig.
All-*t*-retinol	0.2	0.2	0.1	**n.s.**
L*	68.2	73.2	0.9	**
a*	2.4	0.1	0.4	***
b*	13.6	8.8	0.8	***
C^*_{ab}	13.9	8.9	0.8	***
h_{ab}	80.1	90.5	1.8	**
AVI	226.6	93.9	23.2	**

695 s.e.m.; standard error of the means. Sig.: significant differences. ***P<0.01*,
696 ****P<0.001*, n.s.: not significant

697

698

699 **Table 4.** *Liver retinol, retinyl esters (mg/100 g) and mg RE[a]/100 g of foals (Group 3)*
700 *and calves (Group 4) with a diet based on concentrate.*

Compound	Foal	Calf	s.e.m.	Sig.
all-*trans*-Retinol	2.02 (11.8%)	0.42 (7.5%)	0.24	***
Retinyl linolenate	1.60 (9.3%)	0.28 (5.0%)	0.22	***
Retinyl linoleate	2.92 (17.0%)	0.54 (9.6%)	0.37	***
Retinyl oleate	1.12 ± 0.51 (6.5%)	1.00 ± 0.90 (17.8%)	0.16	**n.s.**
Retinyl palmitate	8.14 ± 4.44 (47.6%)	2.08 ± 1.63 (37.1%)	1.06	**
Retinyl stearate	1.34 ± 0.63 (7.8%)	1.29 ± 0.93 (23.0%)	0.17	**n.s.**
RE[a]	10.05 ± 4.95	2.94 ± 1.94	1.18	***

701 s.e.m.: standard error of the means. Sig.: significant differences. ***P<0.01*, ****P<0.001*,
702 n.s.: not significant. The relative percentage of each retinoid relative to the total
703 quantity in brackets

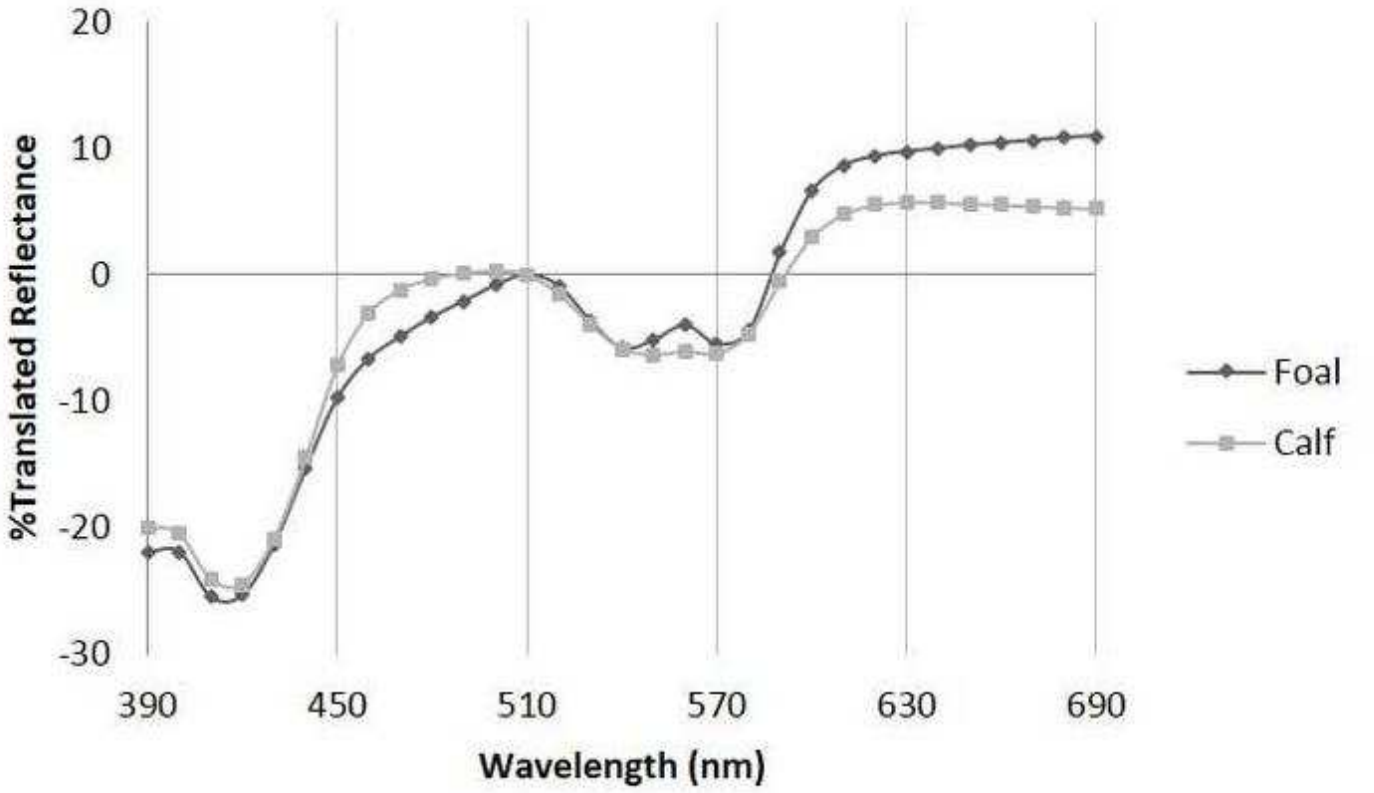
704 [a]mg RE/100 g = mg all-trans-retinol + mg retinyl palmitate/1.83 + mg retinyl oleate/1.92 + mg
705 retinyl stearate/1.93 + mg retinyl linoleate/1.92 + mg retinyl linolenate/1.91.

706

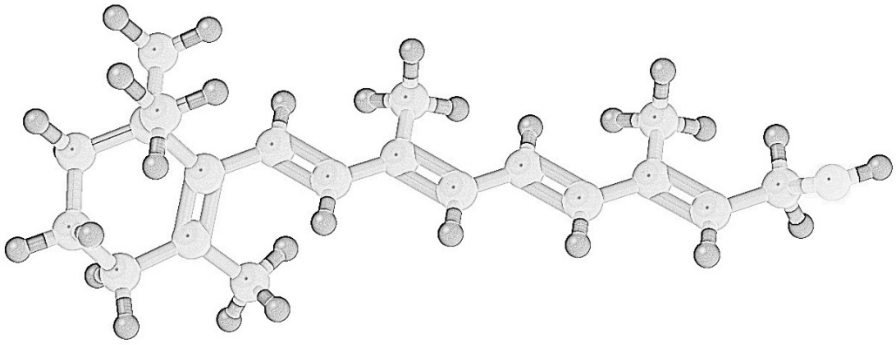
Annex 2: Figures

Figure 1. *Averaged translated reflectance spectra pattern of perirenal fat for foal (group 3) and calf (group 4). The reflectance values have been translated to have reflectance at 510 nm equal to zero.*

Figure
Click here to download high resolution image



GENERAL DISCUSSION



GENERAL DISCUSSION

Diet

Mammals are known to not be able to synthesize carotenoids *de novo* but they can absorb and metabolize some of them when ingested (Schweigert, 1998). Thus, the presence of these compounds in animal biological fluids and tissues depends on the diet. Diets based on pasture are rich in carotenoids, while diets based on concentrate have been reported as poor in carotenoids, because the carotenoids present in the raw material are lost to a great extent due to the manufacturing process (Cardinault et al., 2008; Dunne et al., 2009; Pickworth et al., 2012). In this sense, the results obtained in the experiments carried out in the context of this doctoral thesis are in agreement with the literature, since concentrate diets always showed a poorer carotenoid profile and lower concentrations in comparison to pasture diets. Lutein and β-carotene are reported (Cardinault et al., 2008; Prache et al., 2009) as the main carotenoids in pasture which is in accordance with our results. Besides these two carotenoids, the pastures analyzed showed other minor carotenoids, which content depended mainly on the botanical composition of the pasture, and on the season of sampling, as the carotenoid levels are dependent on climatic and other factors (Figure 12). Violaxanthin appeared in all pasture samples, except in those of the goats's diet (Chapter 1). Zeaxanthin was detected in the pasture of sheep, cattle and horse (Chapters 2 and 4). Antheraxanthin was only present in the grazing sheep´s diet, whilst (*9'Z*)-neoxanthin was found in that of the pig's and ζ-carotene in the pasture of goats. In addition, in the experiment in which the α-tocopherol content was studied (Chapters 3 and 4) that compound appeared in pasture. This was expected since vitamin E is widely distributed in Nature and it is found on the leaves and other green parts of plants (Ramírez & Quiles, 2005). Obviously, retinol was never detected in pasture

samples as this is formed in animals upon the ingestion of pro-vitamin A carotenoids (Ortega et al., 2005).

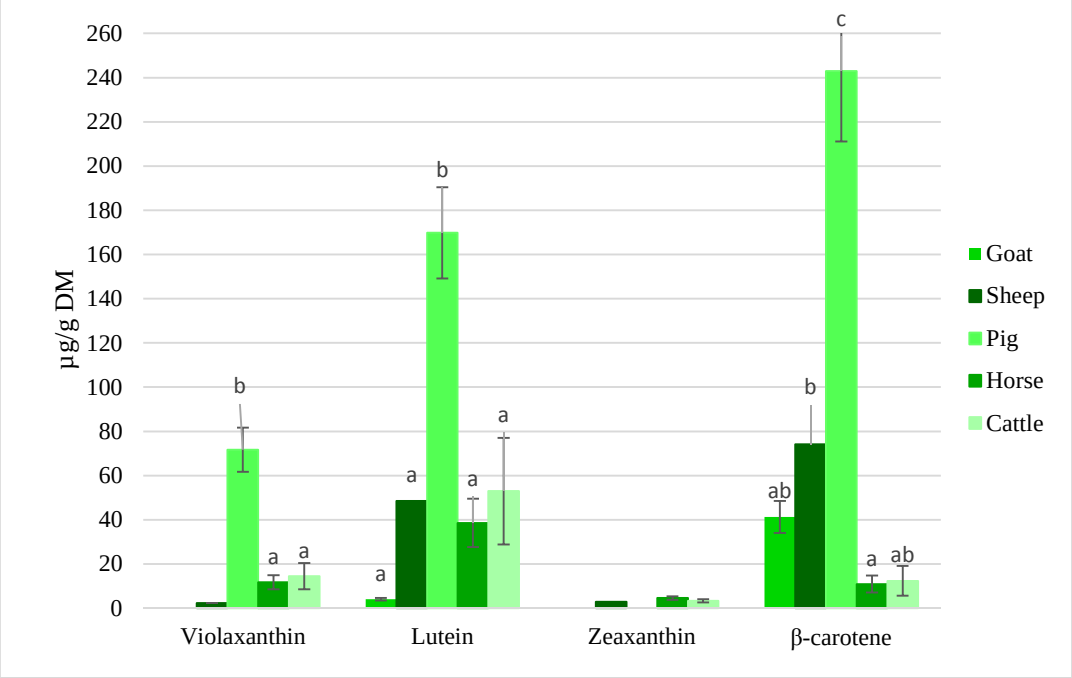


Figure 12. *Main carotenoids (µg/g DM ± S.D.) detected in pastures fed to the different livestock species. Bars with different letters (a, b) were significantly different (P<0.001).*

On the other hand, concentrate diets showed always lutein as the main carotenoid, although in lower levels than pasture (Figures 12 and 13). It is known (Dunne et al., 2009) that manufacturing feeds for concentrates involves processes like heating, grinding, mixing with minerals and pelleting. All these procedures favor oxidation and degradation of carotenoids and fat-soluble vitamins like tocopherol, from primary sources. This explains the low levels of these compounds in all the concentrate feedstuffs analyzed. Even so, other carotenoids were found in some of the concentrate feedstuff as zeaxanthin in concentrate for goats, pigs and cattle or β-carotene in that for goat, cattle and horse livestock (Figure 13). In addition, antheraxanthin and β-cryptoxanthin were detected in

the concentrate for goats and (9Z)-β-carotene in that for cattle. Finally, neither retinol nor α-tocopherol was detected in any of the concentrate diets analyzed.

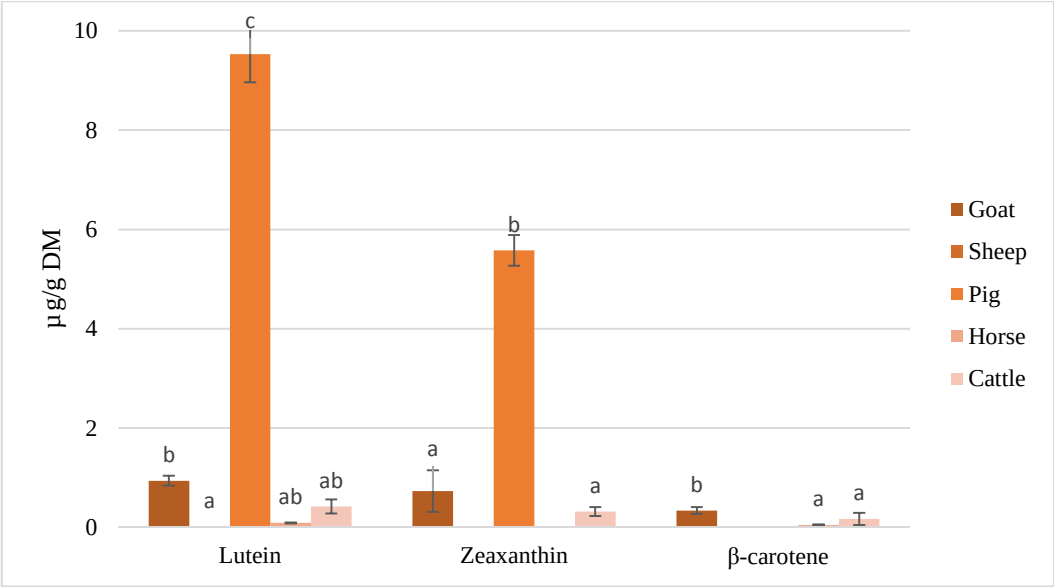


***Figure 13**. Main carotenoids (µg/g DM ± S.D.) detected in concentrates fed to the different livestock species. Bars with different letters (a, b) were significantly different (P<0.001 for lutein and zeaxanthin, P<0.05 for β-carotene).*

To sum up, qualitative and quantitative differences appeared in the carotenoid profile between pastures and concentrates fed to animals among all the experiments of this doctoral thesis. In all cases the carotenoid levels were always higher in pasture than in concentrate.

Plasma

Diet is thought to be the main factor affecting the plasma levels of carotenoids in animals. In this regard animals fed on pasture usually show higher levels in bloodstream than those fed on concentrate (Prache et al., 2003b; Dian et al., 2007a; Dian et al., 2007b;; Serrano et al., 2007; Prache et al., 2009). Besides, species-specific differences in carotenoids absorption and distribution in mammals have been reported (Schweigert, 1998) and, more specifically, between cattle and small ruminants (Yang et al., 1992). Our results quite agree with these previous findings (Figure 14). In this way, lutein was present in the plasma of goats fed on concentrate and pasture but its concentration was higher ($P<0.01$) in the latest group (Chapter 1). This xanthophyll was the only carotenoid present in goat´s plasma which is in agreement with the findings of other studies (Nozière et al., 2006a; Yang et al., 1992). Furthermore, lambs fed on pasture contained both lutein and β-carotene in plasma being, to the best of our knowledge, the first time that β-carotene was reported in lamb´s plasma (Chapter 2). Lambs with a diet based on concentrate did not show any circulating carotenoid because of the absence of these pigments in their diet. Therefore, these results add further support to previous studies (Prache et al., 2003b, 2009) in which the lutein and β-carotene levels are proposed as useful parameters to differentiate lambs fed on pasture from those with a diet based on concentrate and, thus, to be used for feeding traceability purposes.

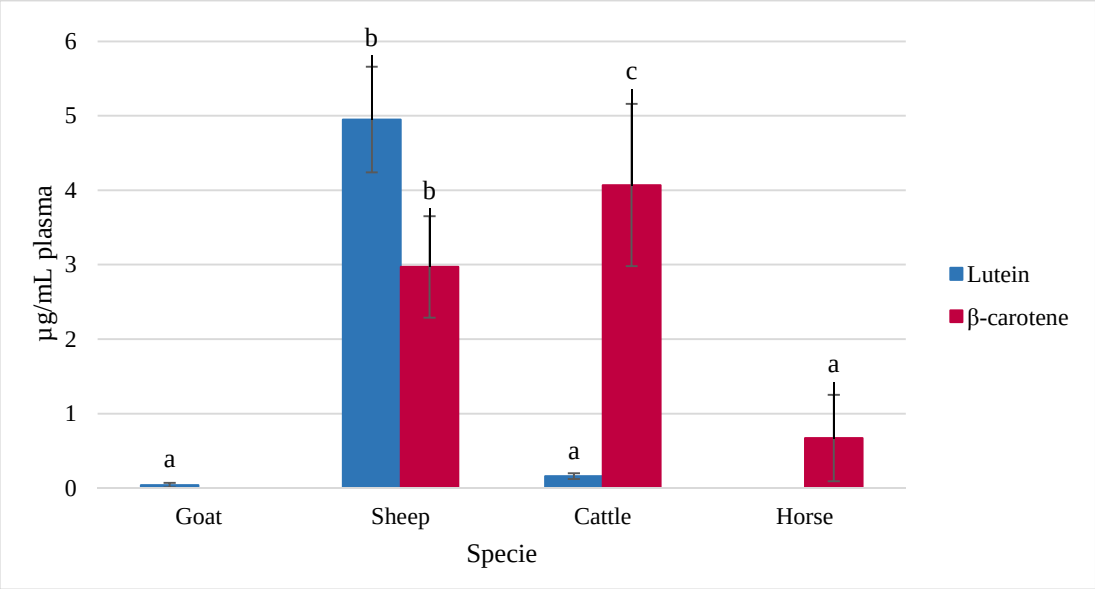


Figure 14. *Lutein and β-carotene levels in plasma (µg/mL ±S.D.) of four livestock species with a diet based on pasture (pig data are not shown due to the absence of carotenoids in their plasma). Bars with different letters (a, b) were significantly different (P<0.001 for lutein and P<0.01 for β-carotene).*

As it has been explained above, differences in the circulating carotenoid profile among ruminants are well-known (Yang et al., 1992). β-Carotene is known to be the main circulating carotenoid in cattle despite the considerable concentration of xanthophylls in its diet (Figure 14). In this sense, cattle fed on pasture (Chapter 4) showed besides lutein, both β-carotene and (*13Z*)-β-carotene in their plasma being their concentration higher than in cattle fed on concentrate. The same was observed for β-carotene in horse´s plasma (Chapter 4) because of an effect of the diet, although in this species lutein was not detected in plasma (Figure 14). This may be due to important differences in the bioavailability of carotenoids between different herbivorous species (Schweigert, 1998). Finally, no carotenoids were detected in pig´s plasma despite their presence in the diet, as already reported (Schweigert, 1998).

On the other hand, retinol was present in the plasma of all the species studied (Figure 15). Significant differences in the concentration of this vitamin in plasma of animals with different feeding system were found within goats groups (*P<0.05*) and within lambs groups (*P<0.01*). That concentration was, for both species, higher in animals with a diet based on concentrate that in animals fed on pasture. This may be attributed to an interaction between α-tocopherol and β-carotene (precursor of retinol) in animals fed on pasture as reported by Yang et al. (2002) who established that, in ruminants, a high concentration of α-tocopherol may interfere with the absorption and metabolism (i.e. its conversion to retinol) of β-carotene. As both compounds are found in lipoproteins in the blood, they may compete with each other for their incorporation into such structures. In this sense, high concentrations of α-tocopherol were found in lambs fed on pasture (Chapter 2), although it was not studied in goat´s plasma.

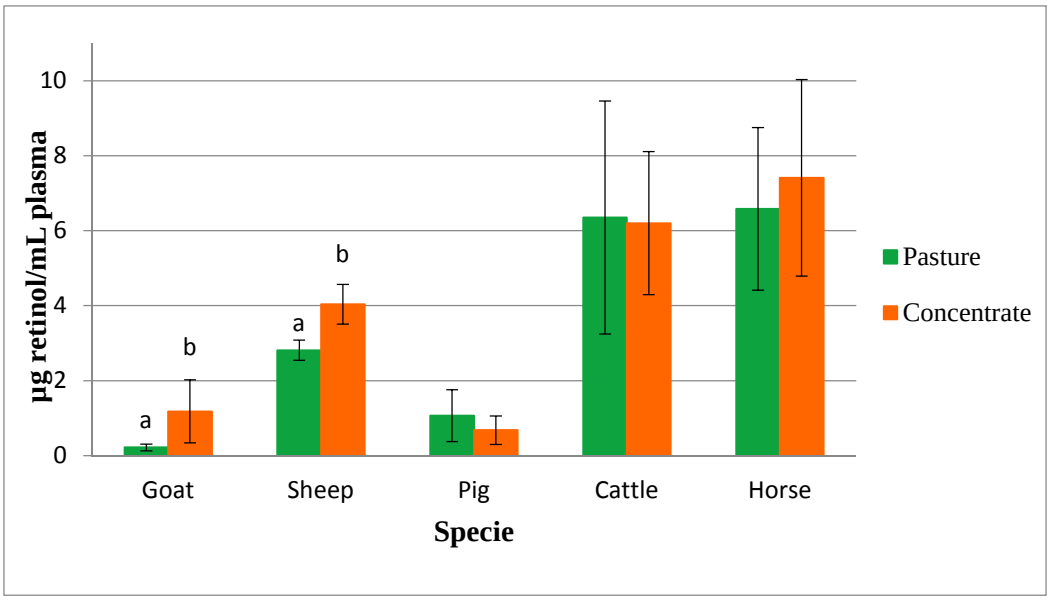


Figure 15. *Retinol levels in plasma (µg/mL ± S.D.) of five livestock species fed on pasture (green) or concentrate (orange). Bars with different letters (a, b) were significantly different (P<0.05).*

With respect to the other three species studied (cattle, pig and horse), the concentration of provitamin A in the diet did not seem to affect the retinol levels in plasma, as no significant differences were found between animals fed on pasture and those fed on a diet based on concentrate within each experiment (Figure 15). These results are consistent with previous studies (Kuhl et al., 2012; Martin et al., 2004) in which it was observed that the levels of vitamin A in blood of mares and cows, respectively, were unaffected by the diet. In relation to this, a homeostatic regulation of vitamin A circulating levels has been pointed out for cattle (Martin et al., 2004). The results obtained in the present doctoral thesis would support this hypothesis about homeostatic regulation of retinol not only in cattle but also in horse.

Adipose tissue

Carotenoids were not detected in adipose tissue in any of the species studied (sheep, cattle, pig and horse). The absence of these compounds in fat from animals with a diet based on concentrate was expectable due to their low levels in the feedstuffs. However, the absence of lutein in the fat of lambs fed on pasture was in disagreement with previous studies (Prache et al., 2003a; Yang et al., 1992) in which this compound was found in the fat of sheep. However, the low persistence of carotenoids in animal´s tissues as well as the extremely low, even negligible, concentration of lutein in sheep fat have been also reported (Prache et al., 2003a; Yang et al., 1992), reasons that could explain the absence of carotenoids in the grazing lambs studied in Chapter 2.

α-Tocopherol appeared in lambs' fat but not in the rest of species, probably due to its absence in the diets. In addition, its concentration in the adipose tissue of lambs fed on pasture was higher than in stall-fed lambs ($P<0.05$) as an effect of the diet. Retinol was

detected in the adipose tissue of the four species studied. Its concentration was affected by the diet in lambs fat, since it was higher (*P<0.01*) in grazing animals. However, in pigs there was not effect of the diet on the concentration of this vitamin, leading us to hypothesize that retinol metabolism in adipose tissue is strongly influenced by the animal species (Figure 16). Additionally, when the deposition of retinol in adipose tissue was compared between foals and calves fed on concentrate (Chapter 4) no significant differences were found (Figure 17), despite significant differences appeared in other tissues. This may indicate that there are certain similarities in the absorption and metabolism of this vitamin in adipose tissue between both species, supporting the hypothesis of a strong influence of specie on the retinol metabolism in adipose tissue of mammals.

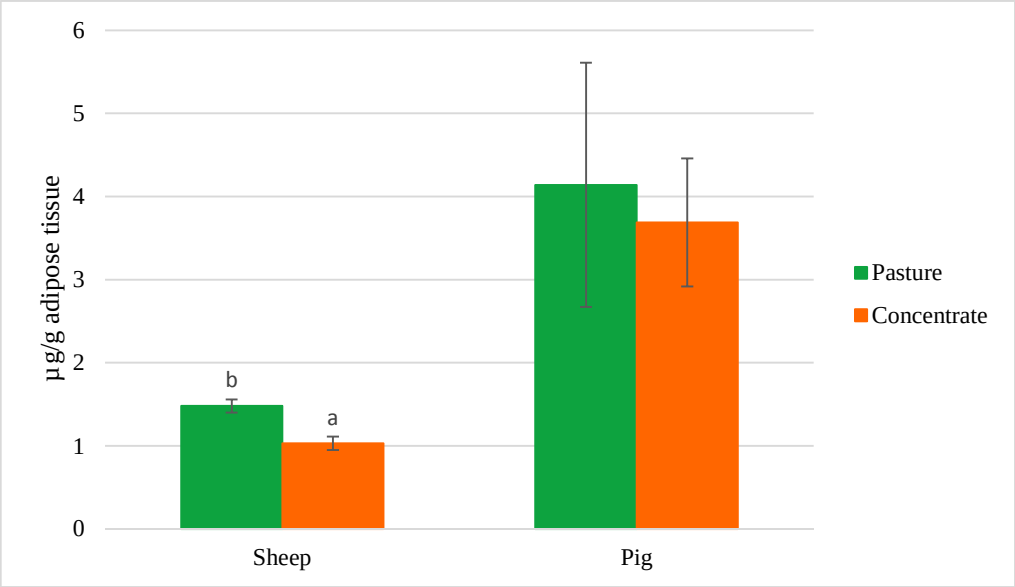


Figure 16. *Retinol levels in adipose tissue (µg/g ± S.D.) of lambs and pigs fed on pasture or concentrate. Bars with different letters (a, b) were significantly different (P<0.001).*

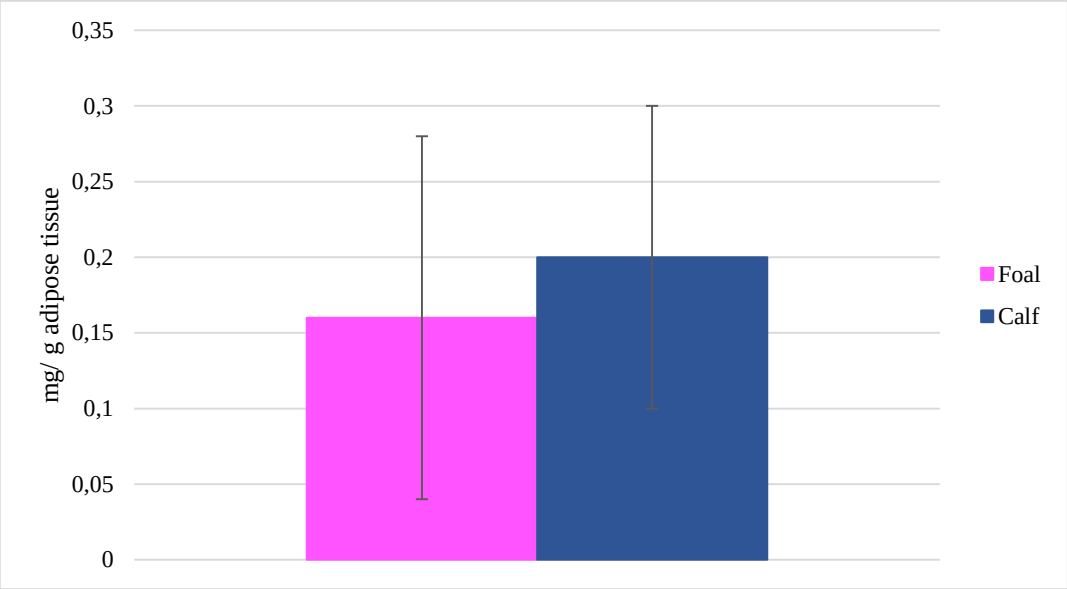


Figure 17. *Retinol levels in adipose tissue (µg/g ± S.D.) of foals and calves fed on concentrate.*

On the other hand, Prache et al. (2003a) concluded that there is a decrease in the concentration of carotenoids in the fat of lambs because of a dilution effect, but they observed that AVI value did not change with this carotenoid decrease. Rather, it remained higher for pasture-fed than for stall-fed animals. This can allow for the differentiation of animals according to their feeding system (extensive *vs.* intensive system). In this way, lambs fed on pasture showed higher ($P<0.001$) AVI values than those with a diet based on concentrate (Chapter 2), so this index allowed to differentiate lambs according to their feeding system despite the absence of carotenoids in their fat, according to the literature (Priolo et al., 2002; Ripoll et al., 2008). Additionally, we have demonstrated that the assessment of retinol and α-tocopherol concentration in adipose tissue together with the AVI index can be harnessed for feeding traceability studies. Nevertheless, when AVI was studied in pigs (Chapter 3) no significant differences were detected according to their feeding system (*Montanera vs. Cebo*). This was in contrast with the ruminant species,

which do absorb carotenoids. AVI values in pig is not affected by the diet so it does not appear as useful from a traceability point of view in this case. Taken together, it seems that the usefulness of the AVI index to classify animals according to their diet may vary from one species to another. For this reason, we decided to compare the AVI indexes of two livestock species (Chapter 4), cattle and horse, reared in the same conditions. They both are known to absorb carotenoids and are classified as "yellow-fat" animals (Schweigert, 1998). The AVI index appeared to be significantly higher ($P<0.01$) in foals than in calves, which could be associated, according to Priolo et al. (2002), to a greater accumulation of carotenoids in foal fat, i.e. a different metabolism and storage of them between both species and, therefore, an important effect of the species on the AVI index.

Liver

It has been reported (Majchrzak et al., 2006; Wingerath et al., 1997) that in mammals up to 80% of the body's total vitamin A is present in the liver. Retinol esterified with different fatty acids (that is, retinyl esters) are the major storage forms (Wingerath et al., 1997), retinyl palmitate being the mainly form of vitamin A in mammals liver. The results obtained in the present doctoral thesis for pigs, cattle and horses agreed well with that observation. Majchrzak et al. (2006) also stated that variations in liver vitamin A concentrations among species were mainly due to different content of retinol or provitamin A carotenoids in the feed supplied to the animals. This matches well with our results, since considering vitamin A (retinyl esters and all-*t*-retinol) in Iberian pigs livers, we were able to differentiate the 92.9% of the animals according to their diet.

Moreover, species-specific differences in the metabolism of vitamin A in liver between herbivores could be presumed, as in the case of carotenoids. To gain further insight into

this, a comparison between cattle and horse was made (Chapter 4). A different profile of retinyl esters was observed (Figure 18) between both species. More specifically, the concentration of most forms of the vitamin A were higher in horse than in cattle (Figure 17). In this regard, Alosilla et al. (2007) reported that vitamin A availability is limited in ruminants due to losses by ruminal destruction. Additionally, to the best of our knowledge, this has been the first time that retinyl linolenate was reported in the liver from horse and cattle.

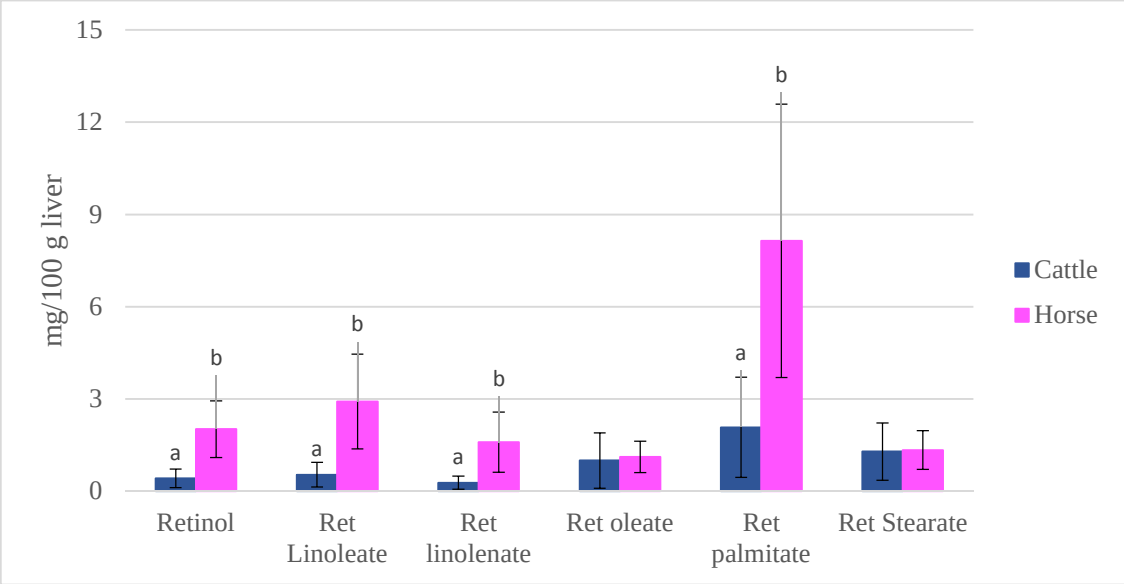


Figure 18. *Retinol profile in liver (mg/100 g ± S.D.) of cattle and horse. Bars with different letters (a, b) were significantly different (P<0.001, P<0.01).*

To sum up, the results obtained seem to indicate that the retinol profile in liver of mammals depends on both the diet supplied to the animals and the species.

Milk

Some milk components like butterfat or fat-soluble compounds are known to be modified by feeding (Gall, 2013; Rafałowski et al., 2014). In this sense, we compared the carotenoids and retinol composition in the milk of goats fed three different diets, namely pasture, concentrate and orange pulp supplementation. Carotenoids were not detected. According to the data reported in the literature (Lucas et al., 2008b) this could be due to a high enzymatic conversion of β-carotene to retinol by the enzyme CCO1 in the intestine of goats. The highest retinol levels in milk were detected in goats with a diet based on pasture and in that receiving orange pulp supplementation with the diet (Chapter 1). Retinol concentration in the milk of animals fed on concentrate was significantly lower ($P<0.001$) in comparison with the other two groups. These observations were in accordance with previous studies (Fedele et al., 2004; Morand-Fehr et al., 2007), where it was reported that the retinol content in milk of goats fed on a grazing system, i.e. a diet rich in carotenoids, was higher (and often significantly) than in milk of stall-fed animals. Thus, the feeding system has an effect on retinol levels in goat milk. Furthermore, milk from goats with a diet rich in carotenoids (pasture or supplemented with orange pulp) would be useful from a nutritional and consumer's point of view, since it shows higher retinol levels than milk from goats with a diet based on concentrate.

On the other hand, as it is well-known, the milk composition varies greatly among livestock species (Gall, 2013). We have compared carotenoid, retinol and α-tocopherol levels in milk from pasture-fed animals among three species: sheep, cow and mare. β-carotene was the only carotenoid detected. It was found in cow and mare milk but not in goat or sheep milk. It has been demonstrated (Mora et al., 2000) that the enzyme CCO1 has lower activity in the intestine of cattle than in that of goat or sheep. This may explain the presence of β-carotene in cow milk that, consequently, appears yellower than that

from the small ruminants. In addition, the levels of β-carotene reported in our study are in consonance with those reported previously (Gentili et al., 2013) in animals fed on pasture and higher than those reported in cows fed on concentrate (Calderón et al., 2007). Moreover, β-carotene concentration was significantly higher ($P<0.05$) in cow than in mare milk (Chapter 4). These findings do not agree with those of other authors (Schweigert & Gottwald, 1999), who found that the concentration of β-carotene in milk of mares were comparable to that of cattle under their experimental conditions.

Retinol was detected in all the milk samples (Figure 19), however no significant differences ($P>0.05$) were found in its levels among species. This fact could be explained in part by the rapid conversion of β-carotene into retinol in small ruminants, as well as a more efficient uptake of plasma retinol by the mammary gland or an ability of that gland to cleave β-carotene into retinol in small ruminants (Calderón et al., 2007). Besides, grazing horses have been reported (Greiwe-Crandell et al., 1997) to develop a poor conversion of provitamin A carotenoids from the diet into retinol. Additionally, as it was explained above, a metabolic regulation of vitamin A is thought to occur in cattle (Martin et al., 2004). All these observations can help understand the absence of significant differences in retinol levels in milk among species.

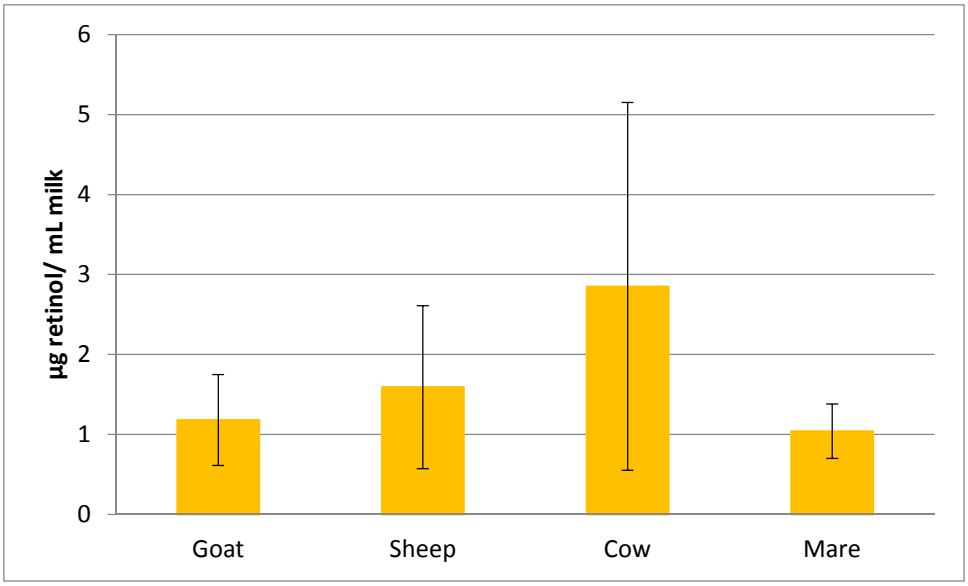


Figure 19. *Retinol levels in milk (µg/mL ± S.D.) from goat, sheep, cow and mare fed on pasture.*

Finally, α-tocopherol has been reported (Gentili et al., 2013) as the main source of vitamin E in milk from different species. These authors reported the highest levels of this compound in goat milk, which is not in accordance with the data obtained under our experimental conditions (Figure 20). In our study, under our trial conditions, α-tocopherol levels in milk were significantly ($P<0.001$) higher in cow than in goat and sheep. Moreover, this vitamin was not detected in mare milk (Figure 19). In this sense, Engel et al. (2007) found a high depletion (3x) of α-tocopherol levels in mares from colostrum to milk until achieving minimal levels of this vitamin at 4 days of lactation. This could explain to some extent the absence of this vitamin in mare milk.

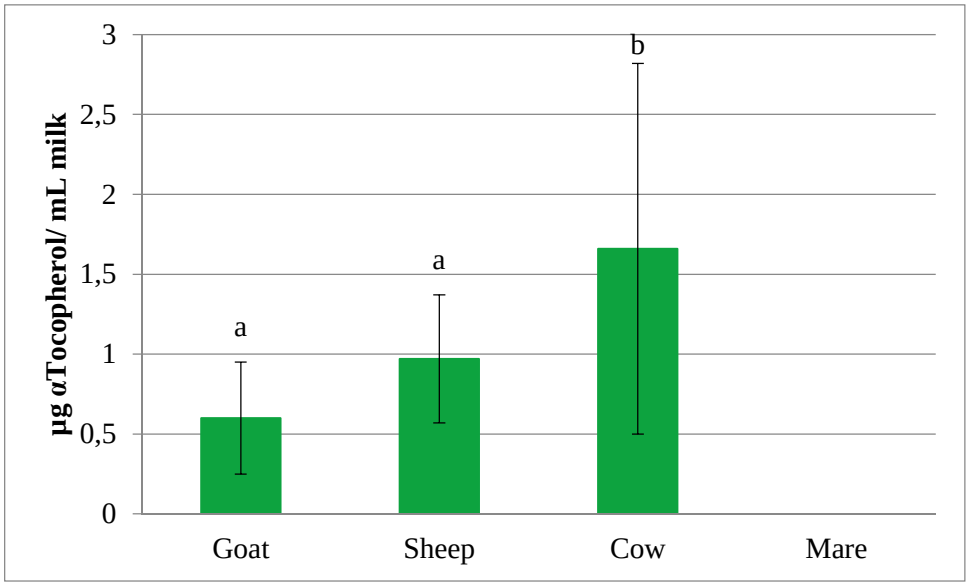
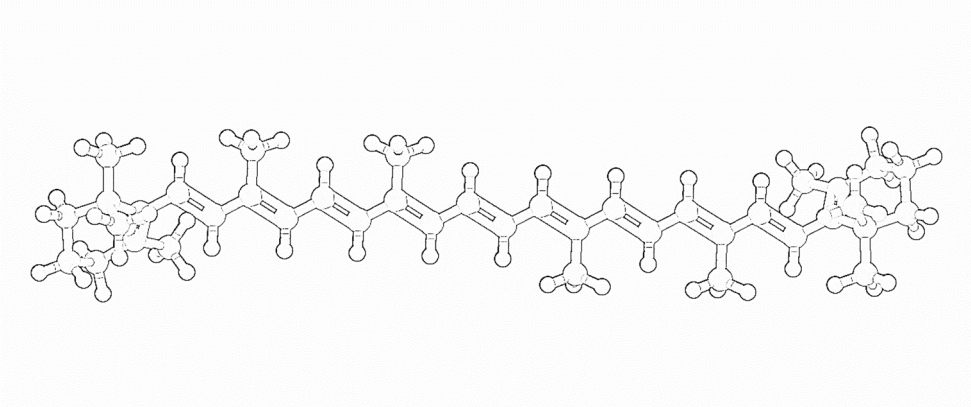


Figure 20. *α-Tocopherol levels in milk (µg/mL ± S.D.) from goat, sheep, cow and mare fed on pasture. Bars with different letters (a, b) were significantly different (P<0.001).*

CONCLUSIONS



CONCLUSIONS

FIRST

Retinol levels in milk from goat fed on a diet rich in carotenoids (based on pasture or supplemented with orange pulp) were higher ($P<0.001$) than retinol levels in milk from goats on a diet based on concentrate. In this sense, orange pulp supplementation in goats may be interesting from a nutritional point of view and in the context of the valorization of by-products of the citrus industry.

SECOND

Milk from goats fed on pasture showed higher ($P<0.001$) n-3 fraction and CLA (total Conjugated Linoleic Acid) *cis*-9, *trans*-11 (Rumenic Acid) than milk from the other two groups (fed on concentrate or supplemented with orange pulp) which is a healthier FA profile.

THIRD

Lutein and β-carotene were detected in the plasma of lambs fed on pasture but not in those fed on concentrate or in suckling lambs, therefore these compounds could be propose as discriminant parameters for feeding systems in lambs.

FOURTH

Higher levels of retinol ($P<0.01$) and α-tocopherol ($P<0.05$) were detected in plasma of lambs reared on an extensive system than in those reared on an intensive system. Therefore, a rearing system based on pasture can improve the nutritional

value of sheep products, since the higher levels of retinol and α-tocopherol that it contains can be transferred to sheep tissues.

FIFTH

The combined used of AVI, retinol and α-tocopherol levels in perirenal fat of lambs allows a good discrimination according to the diet (pasture vs. concentrate). Thus, these parameters are proposed as good tools for feeding traceability studies in sheep livestock.

SIXTH

The rearing system of Iberian pigs (*Montanera* and *Cebo*) do not affect the plasma and perirenal fat retinol contents.

SEVENTH

Retinoids content in liver, as well as L* and h_{ab} values in perirenal fat in Iberian pigs appeared as useful parameters to differentiate these animals according to their feeding system. Consequently, they may be interesting for traceability purposes.

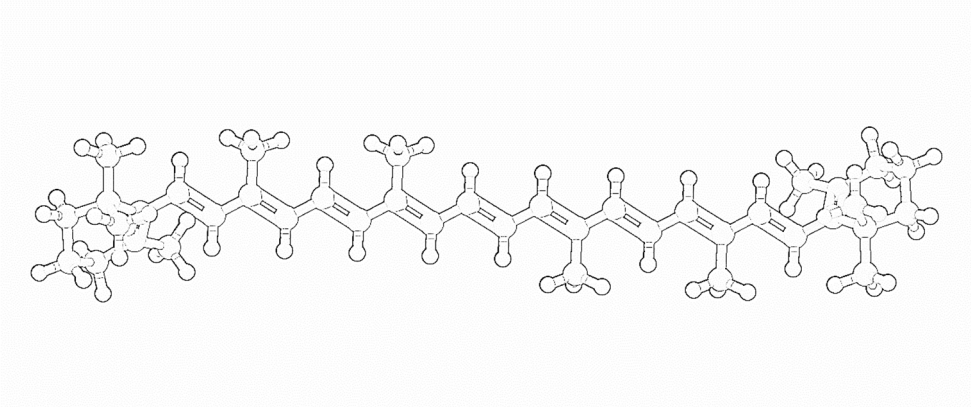
EIGHTH

Under the same rearing conditions, carotenoids levels in plasma ($P<0.001$) and milk ($P<0.05$) were higher in cows than in mares, but more studies are needed in order to establish the carotenoids metabolism in horses.

NINTH

Under the same indoor rearing conditions, the foal seems to accumulate retinoids in liver to a greater extent than calf so foal's liver can be considered a good source of vitamin A which is interesting from a nutritional point of view.

CONCLUSIONES



CONCLUSIONES

PRIMERA

Los niveles de retinol en leche de carbas alimentadas con una dieta rica en carotenoides (basadas en pastos o suplementada con pulpa de naranja) fueron mayores ($P<0,001$) que aquellos en leche de cabras con una dieta basada en alimentos concentrados. En este sentido, la suplementación de la dieta de las cabras con pulpa de naranja parece interesantes desde un punto de vista nutricional y en el contexto de la revalorización de los subproductos de la industria de los cítricos.

SEGUNDA

La leche de cabras alimentadas con pasto presentó mayores niveles ($P<0,001$) de la fracción n-3 y de *cis*-9 CLA (Ácido Linoleico Conjugado total) y *trans*-11 (Ácido Rumenico) que la leche de los otros dos grupos (animales alimentados con concentrados o con una dieta suplementada con pulpa de naranja), lo cuál supone un perfil de ácidos grasos más saludable.

TERCERA

Se detectó luteína y β-caroteno en el plasma de los corderos alimentados con pasto pero no en aquellos alimentados con concentrados o en los corderos lactantes, por tanto estos compuestos podrían proponerse como parámetros útiles para discriminar los sistemas de alimentación en corderos.

CUARTA

Se detectaron mayores niveles de retinol (*P<0,01*) y α-tocoferol (*P<0,05*) en el plasma de los corderos criados en un sistema extensivo que en aquellos criados en un sistema intensivo. Por lo tanto, un sistema de producción basado en pastos puede mejorar el valor nutricional de los productos ovinos, ya que la concentración circulante de ambas vitaminas puede transferirse a los tejidos del animal.

QUINTA

El uso combinado de los valores de AVI con los niveles de retinol y α-tocoferol en grasa perirenal de corderos con una alimentación basada en pastos o en alimentos concentrados permite una buena discriminación de los animales de acuerdo a su dieta. Por ello, se proponen estos parámetros como herramientas útiles en estudios de trazabilidad alimentaria en ganado ovino.

SEXTA

El sistema de producción en cerdos ibéricos (*Montanera* y *Cebo*) no afecta al contenido en retinol del plasma y la grasa perirenal de estos animales.

SÉPTIMA

Tanto el contenido en retinoides en hígado como los valores de L* y h_{ab} en grasa perirenal en cerdos ibéricos son parámetros útiles para diferenciar estos animales según su sistema de alimentación y, por tanto, para ser utilizados en estudios de trazabilidad

OCTAVA

Bajo las mismas condiciones de producción, los niveles de carotenoides en plasma y leche fueron mayores ($P<0,001$ y $P<0,05$, respectivamente) en vacas que en yeguas, pero son necesarios más estudios para establecer el metabolismo de los carotenoides en caballos.

NOVENA

Bajo las mismas condiciones de producción, el potro parece que acumula retinoides en el hígado mejor que el ternero por lo que el hígado de potro podría ser considerado como buena fuente de vitamina A, lo cual es interesante desde un punto de vista nutricional.

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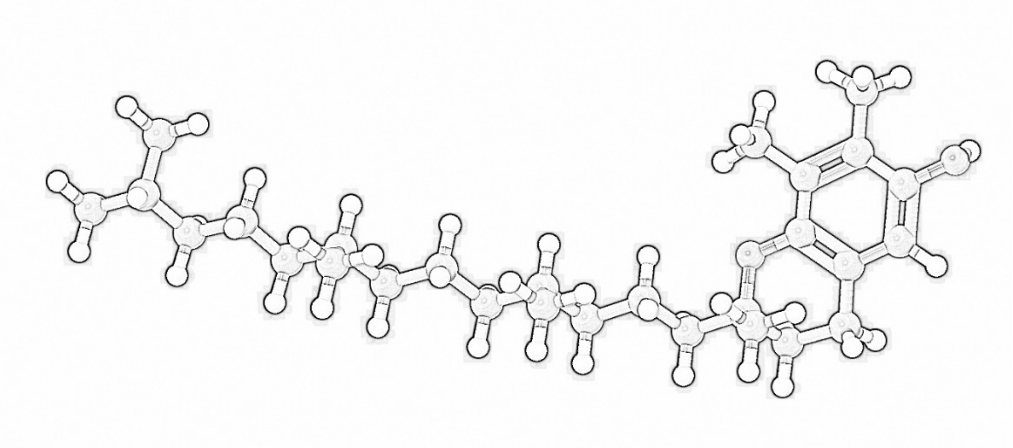

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